Expression and role of PKC in control of excitation-contraction coupling in ureter smooth muscle

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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ABSTRACT

The aim of this work was to investigate possible role of PKC in control of excitation-contraction coupling in phasic guinea pig and rat ureter smooth muscle. Immunohistochemistry and western blotting were used to identify the expression of four PKC isoforms α, β, δ and ε in ureter smooth muscle of both species. Photometric system combined with force measurement and electrical activity as well as confocal imaging of isolated ureteric myocites have been used in the functional studies. The effects of PKC activator PDBu and PKC inhibitor Ro320432 on force and Ca^{2+} induced by different modes of stimulation of ureter smooth muscle have been examined. It is established that expression and functional effects of PKC activation and inhibition in ureter smooth muscle were species dependent. Guinea pig ureter smooth muscle expressed predominantly PKC β and δ while rat ureter showed the presence of one isoform PKC α. Direct activation of PKC by PDBu produced an increase in the amplitude of force which in the guinea pig ureter was associated with an increase in the duration of the Ca^{2+} transient. These changes in Ca^{2+} and force were associated with an increase in the duration of the plateau component of the action potential. The data obtained suggest that the prolongation of the plateau component of the action potential by PDBU in the guinea pig ureter smooth muscle was not associated with inhibition of Ca^{2+} sparks/STOCs coupling mechanism or modulation of the activity of the Na^{+}-Ca^{2+} exchanger but is likely to be produced by stimulation of the Ca^{2+} inward current via L-type Ca^{2+} channels. In the rat ureter smooth muscle an increase in the amplitude of force was totally Ca^{2+}-independent and was associated with sensitization of the contractile machinery to Ca^{2+} via inhibition of MLCP activity. In both guinea pig and rat ureter PKC is partly but not fully responsible for the stimulant action of agonists.

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Abbreviations

4-AP	4-aminopyridine
AP	Action Potentials
ATP	Adenosine 5-triphosphate
ATPase	Adenosine triphosphatase
AM	Acetoxymethyl ester
Ba^{2+}	Barium ion
BayK8644	Calcium channel agonist
BK_{Ca}	Large conductance calcium activated potassium channel
Ca^{2+}	Calcium ion
Ca-ATPase	Calcium adenosine triphosphatase
$[Ca^{2+}]_i$	Cytosolic calcium concentration
CaM	Calmodulin
CaM kinase II	Calmodulin-dependent protein kinase II
CCh	Carbachol
CICR	Calcium induced calcium release
Cl_{Ca}	Calcium activated chloride channel
cm	Centimetre, length
CPA	Cyclopiazonic acid
CPI-17	Protein kinase C activated myosin phosphatase inhibitor
DAG	Diacylglycerol
DDT	Dithiotheridol
DMSO	Dimethylsulphoside
ECL	Enhanced chemiluminecence
EGTA	Ethylenediaminetetraacetic acid
Hz	Herts
IICR	IP3-induced Ca release
IP_3	Inositol(1,4,5)-tris-phosphate
IP_3R	Inositol(1,4,5)-tris-phosphate receptor
K^+	Potassium
$[K^+]$	Potassium concentration
K_{ATP}	ATP sensitive potassium channel
KDa	KiloDalton
K_{IR}	Inward rectifier potassium channel
L	Litre
L-type Ca channel	Calcium channel
M	Molar
mA	milli Amp
mg	milli-gram
$MgCl_2$	Magnesium Chloride
min	minute
ml	milliliter
MLCK	Myosin light chain kinase

MLCP	Myosin light chain phosphatase
MLC_{20}	20 kDa Myosin light chain
mm	millimeter
mM	millimolar
mol	moles
mN	millinewton
ms	millisecond
MYPT	Myosin phosphatase target subunit
MV	millivolt
n	Sample size
N	Newton
Na	Sodium
nM	nanomolar
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMCA	Plasma membrane Ca-ATPase
RyRs	Ryanodine receptor
ROK	Rho-associated kinase
s.e.m	Standard error of the mean
SERCA	Sarcoplasmic reticulum Ca-ATPase
SR	Sarcoplasmic reticulum
STIC	Spontaneous transient inward current
STOC	Spontaneous transient outward current
TEA	Tetraethylammonium
TTX	Tetrodotoxin
w/v	Weight per volume

Chapter 1

Introduction

Chapter 1

Introduction

1 Physiology and morphology of the ureter

1.1 Structure and function of the ureter

The ureter is a fibro-muscular tube connecting the bladder and kidney and is responsible for the transportation of urine from the kidney to the urinary bladder. Generally, the ureter helps to maintain kidney function by keeping the hydrostatic pressure of the renal collecting system low.

There are two ureters connecting with two kidneys in the body (Figure 1.1). Each ureter originates at the renal pelvis of the kidney, and terminates at the ureterovesical junction. This junction prevents retrograde urine reflux from the bladder into the ureter. The activity of ureteric smooth muscle is myogenic. Therefore, its contraction is not initiated by nerve stimulation or hormone regulation but by itself. The influence of the autonomic nervous system is secondary to the myogenic function and it contributes to the modulation of contractile activity. The ureter from most of the mammalian animals displays spontaneous contractile activity *in vitro* if the pelviureteral junction is kept intact (Exintaris *et al.*, 1999). Some studies carried out on human ureter *in vitro* suggest that spontaneous activity has neural and hormonal input (Cole *et al.*, 1988; Hua *et al.,* 1987; Exintaris *et al.*, 1999).

The ureter is a fibro-muscular tube consisting of a lining of urothelium, a layer of

connective tissue outside the smooth muscle cells which are arranged into interconnecting muscle bundles. These bundles have different thickness and orientation so that excitation can pass along the muscle networking without the need for nerves (Figure 1.2). The individual smooth muscle fibers are 5μm wide and 100 -150μm long. The smooth muscle bundles spread differently along the different portions of the ureter (Tachibana *et al.*, 1985). In the proximal and distal renal pelvis the arrangement of ureteric smooth muscle cell is loosely packed (Klemm *et al.*, 1999). In the middle portion of ureter, the smooth muscle bundles are randomly oriented in an irregular manner. In the lower portion of the ureter, the bundles are longitudinal oriented (Tachibana *et al.*, 1985).

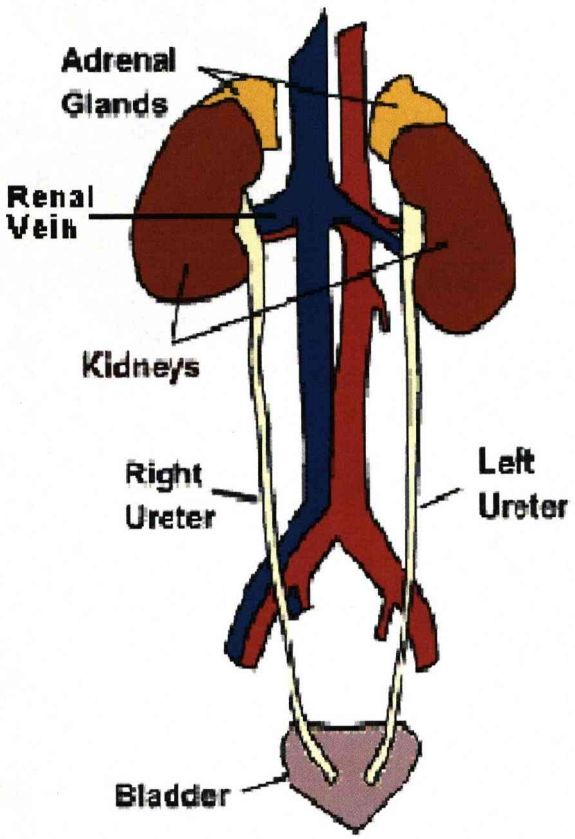


Figure 1.1 Overview of urinary system. Two ureters connecting kidneys and bladder.

(Figure modified from www.biologycorner.com/.../rat_urogenital.html).

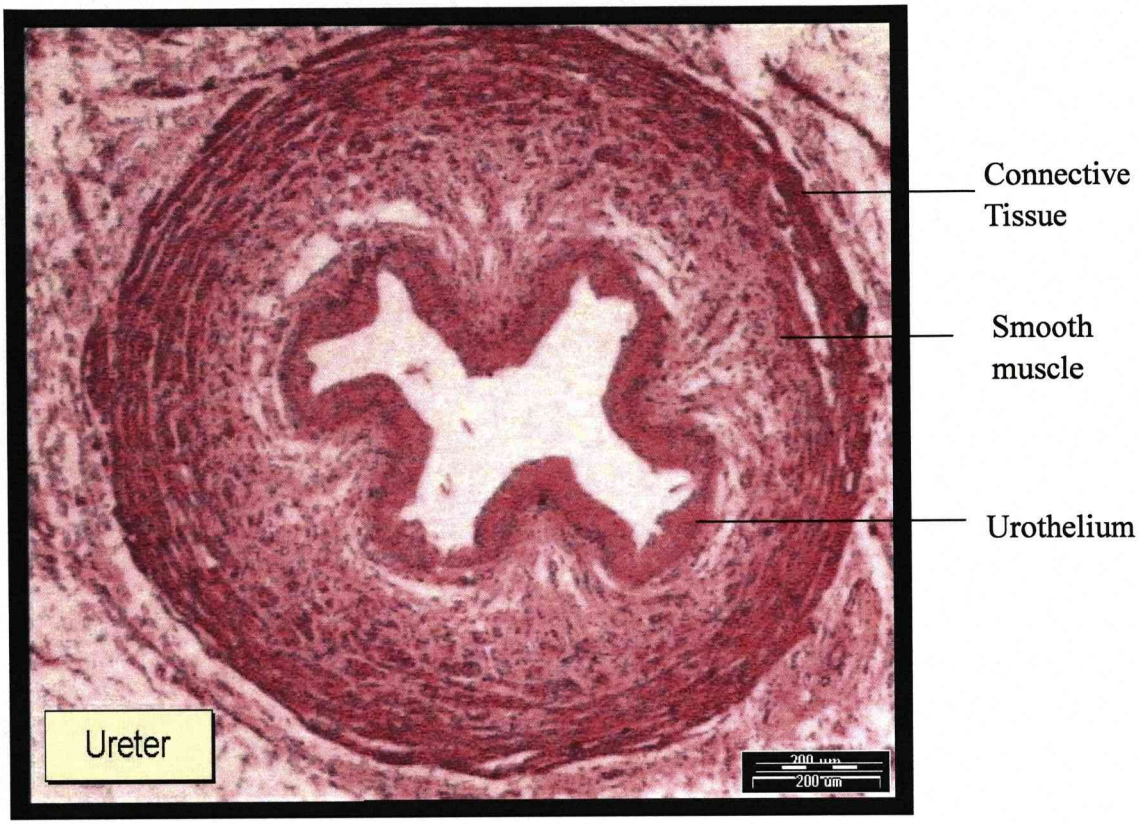


Figure 1.2 Cross section of human ureter showing ureteric morphology. (Figure modified from www.technion.ac.il/~mdcourse/274203/lect14.html).

1.2 Peristalsis of the ureter

The ureter propels urine in a peristaltic manner. Peristalsis of the ureter is autonomous and can be activated by stretch induced by increased urine volume. The peristaltic activity, which is controlled by a propagating action potential, originates from pacemaker cells at the proximal portion of the renal pelvis. Ureteric smooth muscle cells are normally quiescent and do not generate peristaltic activity unless being signaled by pacemaker cells. The action potential discharged from multiple pacemaker cells summates to trigger a wave of electrical activity which passes distally along the smooth muscle cells into the main ureter and gives rise to the mechanical event of peristalsis. This mechanism allows the ureter to propel a bolus of urine from kidney to the urinary bladder. Normal peristaltic activity of ureter smooth muscle could be disrupted by deficiencies in conduction of the coordinated contraction wave. It can be caused by inflammation and some other pathological problems like ureteric obstruction and reflux. Medical problems of the ureter also include ureteric stone, urethritis, cancer, and ureterocele.

1.3 Ureteric cells

Smooth muscle cells are the main functional unit of the ureter. So far, three types of cells that have been identified in ureter on the basis of their morphological and functional characteristics. They are termed atypical smooth muscle cells, typical smooth muscle cells and ICC-like interstitial cells.

1.3.1 Pacemaker cells and their activity

It has long been accepted that myogenic contractile activity arises from the electrically active atypical smooth muscle cells localized in the proximal regions of the upper urinary tract. This region has similar properties to the sino-atrial node of the mammalian heart which is characterized by irregular cell shapes and contents (Gosling & Dixon, 1972; Lang *et al.*, 2006, 2007 (1)). The atypical smooth muscle cells generate a simple pacemaker action potential which appears as a single plateau type action potential (Klemm *et al.*, 1999).

The distribution of atypical smooth muscle cells has been identified. They are located mostly at the renal pelvis of the mammalian ureter and their distribution decreases with distance from the proximal region and is nearly absent in the region of ureteropelvic junction (UPJ) (Lang *et al.*, 1998, 2001). More than 80% of the smooth muscle cells in the pelvi-calyceal junction (PCJ) and 15% of the smooth muscle cells in the proximal renal pelvis were atypical smooth muscle cells. No atypical smooth muscle cells were found in the ureter proper and distal regions of the renal pelvis (Klemm *et al.*, 1999). This is consistent with the distribution of action potentials recorded from pacemaker cells along the PCJ, the proximal renal pelvis, the distal renal pelvis and the ureter. In addition, the decreased distribution of atypical smooth muscle cells along the renal pelvis compared to the UPJ is consistent with the myogenic activity found in renal pelvis but absent from the end of ureter (Lang *et al.*, 2002). Furthermore, an ureteropelvic junction, dissected free of ureter can maintain its

myogenic activity producing spontaneous contractile activity (Lang and Zhang, 1996). Above all, this suggests that atypical cells exert pacemaker cell activity. In rat, it has been shown that the atypical smooth muscle cells can directly drive typical smooth muscle cells (Lang *et al.*, 2002).

Atypical smooth muscle cells are short spindle-shaped and form a thin layer of loosely arranged cells (Gosling & Dixon, 1974). Their shapes and cell contents differ from the typical smooth muscle cells. Atypical smooth muscle cells have smaller nucleus lack non-specific cholinesterases (Gosling & Dixon, 1972, 1974; Klemm *et al.*, 1999).

1.3.2 Other smooth muscle cells

Recently, it has been suggested that, in addition to atypical smooth muscle cells, ICC-like cells also play the role of pacemaker cells, but secondary to the role of atypical smooth muscle cells, and display spontaneous electrical activity (Lang *et al.*, 2006). ICC-like cells identified in the renal pelvis and the pelvi-calyceal junction display many characteristics which relate them to classical interstitial cells of Cajal (ICC) originally found in the intestine. Furthermore, they perform numerous communicating interconnections with other smooth muscle cells (Burns *et al.*, 1997). ICC-like cells were oval-shaped and have a nucleus about 3-5 μm wide. ICC-like cells generate an intermediate action potential consisting of a single spike and a non-oscillatory plateau component.

Typical smooth muscle cells are the predominant cells in the main ureter. The smooth muscle cells are tightly arranged into bundles and create a compact layer of muscle wall (Klemm *et al.*, 1999). The action potential recorded from the typical ureter smooth muscle cell is a driven action potential which consists of an initial spike component followed by an oscillatory plateau component (Shuba, 1977(2); Klemm *et al.*, 1999).

2 Mechanism of excitation-contraction coupling

In ureteric smooth muscle cells, the elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is the main trigger for contraction. An increase in $[Ca^{2+}]_i$ is either due to Ca^{2+} influx into the cell during depolarization or Ca^{2+} release from SR. Ca^{2+} entry into the cell is the main mechanism for the increased intracellular Ca^{2+} concentration in ureteric smooth muscle during the generation of the action potential (Sui *et al.*, 1997). It has been found that in ureter smooth muscle Ca^{2+} influx into the cells is mediated not only by the voltage-dependent L-type Ca^{2+} channel, but also on the Na^{+}-Ca^{2+} exchanger in "Ca^{2+} entry" mode (Aickin *et al.*, 1984).

2.1 The role of the action potential in ureteric smooth muscle

The action potential produced in ureteric smooth muscle consists of a series of spikes followed by a prolonged plateau component lasting hundreds of milliseconds with oscillations of potential and a rapid declining repolarization phase terminated by an after-hyperpolarization (Figure 1.3). The major ionic currents underlying the action potential in the guinea pig ureter smooth muscle are Ca^{2+} inward current through voltage-activated L-type Ca^{2+} channels and Ca^{2+}-activated K^{+} current which is the main outward current. 4-AP sensitive transient K^{+} current is also present.

The action potential recorded from single cells is similar to that seen in the multicellular preparation (Imaizumi *et al.*, 1989). Under voltage clamp conditions, with KCl in the pipette solution, depolarization steps from a holding potential of

–60mV to 0mV triggers a complex ionic current consisting of transient inward Ca^{2+} current followed by oscillatory K^+ outward current (Lang, 1989).

The action potential can not be abolished by neurotoxins such as terodotoxin and capsaicin, which suggests that the generation and propagation of the action potential is myogenic (Washizu, 1966; Maggi *et al.*, 1995). However, the action potential can be abolished by Ca^{2+} channel blockers nifedipine, Cd^{2+} and Co^{2+}. There is an absolute requirement for Ca^{2+} to generate the action potential. In addition, the elimination of extracellular Na^+ can also abolish the plateau component of the action potential (Kuriyama & Tomita, 1970; Shuba, 1977(2)). It has been suggested that Na^+ entry via a voltage-dependant Na^+ channel which is not sensitive to tetrodotoxin, is involved in generation of plateau component of the action potential (Shuba, 1977(2)). Na^+ entry may be important for the maintenance of the plateau phase of the action potential. BK_{Ca} channels were reported to play a key role in control of the duration of the plateau component of the action potential (Imaizumi *et al.*, 1989).

Initiation of the action potential is mainly due to the activation of L-type Ca^{2+} channels resulting in Ca^{2+} influx into the cell, which generates the Ca^{2+} inward current. The initial spike component observed is due to the rapid activation of Ca^{2+} channels. Elevation of the extracellular Ca^{2+} concentration increases the amplitude of the initial spike (Vereecken *et al.*, 1975; Exintaris *et al.*, 1999). After the initial depolarization, a steady state - plateau component follows, during which oscillations are observed. This

has been suggested to be due to the slow inactivation of the Ca^{2+} inward current and repetitive activation of the Ca^{2+}-activated K^+ current. A slowly inactivating inward Ca^{2+} current counteracts the activated transient outward K^+ current to generate the long lasting plateau component of the action potential (Imaizumi *et al.*, 1989). Subsequently, repolarization of the action potential is caused by gradual activation of K^+ channels overcoming the inward Ca^{2+} current repolarizing the cell to the resting membrane potential (Imaizumi *et al.*, 1989). Finally, smooth muscle cells can not be activated during the hyperpolarization phase. When the ureter was stimulated *in vivo* by electrical stimulation at a slower rate than that of spontaneous activity generated by pacemaker cells, the contractions are inconsistent. This irregular frequency is due to the fact that the electrical stimuli are applied during the refractory period of the spontaneous activity (Weiss *et al.*, 2006). The 4-AP sensitive K^+ current has been suggested to be involved in the hyperpolarization and refractory period (Exintaris *et al.*, 1999). The Ca^{2+} sparks/ STOCs coupling mechanism was shown to play a leading role in the generation of the refractory period in guinea pig ureteric smooth muscle (Burdyga & Wray, 2005).

The action potentials of ureteric smooth muscle are species dependent. The above description is mainly based on the studies carried out on the guinea pig ureteric smooth muscle. In rat ureter, the action potential and the ionic currents underlying this mechanism are different. The action potential is characterized by an initial fast spike followed by a plateau component without oscillations on the plateau phase. Unlike

guinea pig ureter smooth muscle, five ionic currents have been found that contribute to the generation of the action potential in rat ureter- two inward currents; a Ca^{2+}-activated Cl^- current and a Ca^{2+} current; and three outward currents- a Ca^{2+}-dependent K^+ current, an A type Ca^{2+}-independent K^+ outward current and a delayed rectifier type K^+ current (Smith *et al.*, 2002). In human ureter, the action potential is similar to the action potential observed in rat ureter (Figure 1.4) (Santicioli & Maggi, 1998).

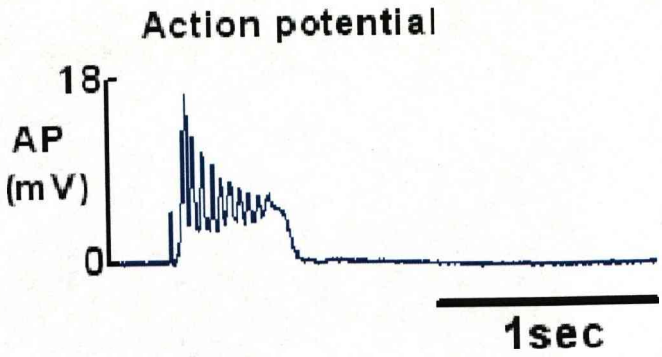


Figure 1.3 A representative action potential of guinea pig ureter. The action potential consists of a series of spikes and a prolonged plateau component (Figure modified from Burdyga & Wray, 1999).

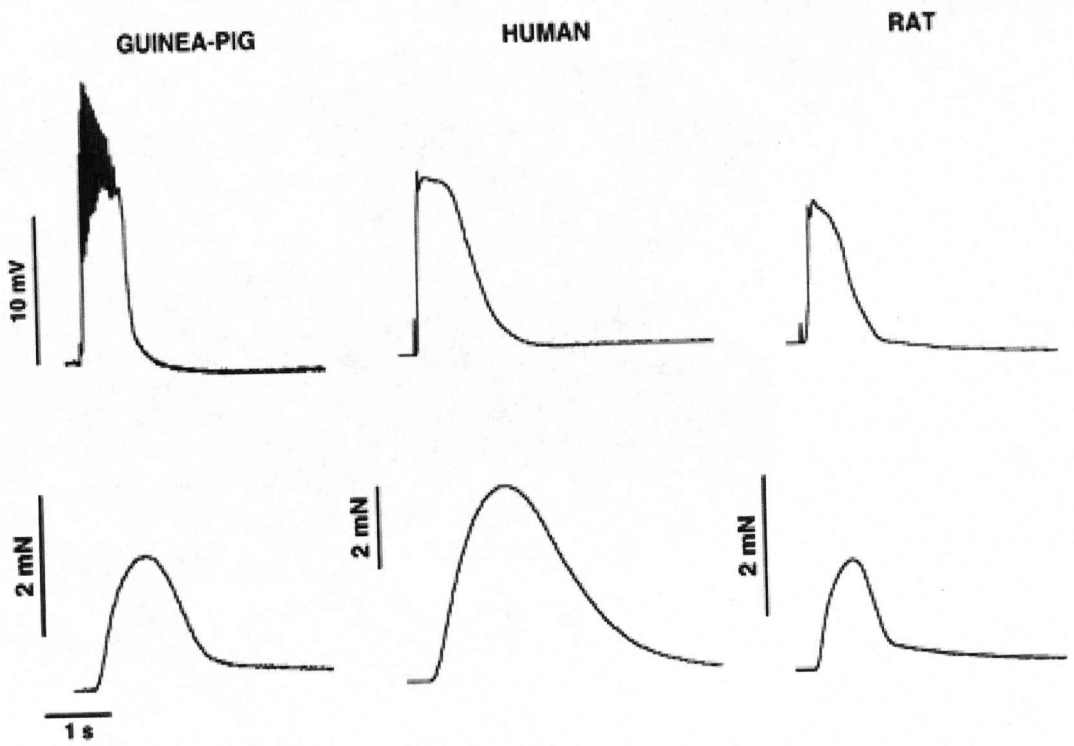


Figure 1.4 Action potential and phasic contraction recorded from guinea pig, human and rat ureter smooth muscle (Figure modified from Santicioli and Maggi, 1998).

2.1.1 The Ca^{2+} currents

It has been widely accepted that an inward Ca^{2+} current via L-type Ca^{2+} channels is the major depolarizing current to initiate the action potential in smooth muscle (Bolton et al., 1985; Ganitkevich et al., 1986). Several characteristics of the inward Ca^{2+} current have been identified in ureteric smooth muscle. In both rat and guinea pig ureter, the Ca^{2+} current can be completely abolished by the Ca^{2+} channel blockers nifedipine and verapamil, which results in a decreased duration and amplitude of the action potential and eventually full abolition of the action potential.

In the guinea pig ureteric smooth muscle, the Ca^{2+} current is activated at potentials positive to –50 mV. Peak amplitude reaches between 0 and +10 mV (Lang, 1989; Sui, *et al.*, 1997). The Ca^{2+} current is inactivated slowly and is suggested to account for the sustained membrane depolarization during the long duration action potential by opposing the outward current (Sui, *et al.*, 1997). The activation of the L-type Ca^{2+} channel by BayK8644 enhances the inward Ca^{2+} current which leads to an increased outward current in guinea pig ureteric smooth muscle (Lang, 1989; Sui, *et al.*, 1997).

2.1.2 K^+ currents

Generally, there are three kinds of outward currents found in excitable ureteric smooth muscle cells that can be resolved using voltage-clamp and a various pharmacological tools. These include a K^+ current activated by intracellular Ca^{2+} (I_{KCa}), a voltage dependant but not Ca^{2+} dependent K^+ current (I_{to}) and a delayed rectifier type of K^+ current (I_{Kr}) (Smith *et al.*, 2002).

In guinea pig ureter smooth muscle, two types of outward potassium currents have been observed upon depolarization: The dominant K^+ current (I_{Kca}) elicited by depolarization has a large conductance and is carried by Ca^{2+}-activated K^+ (BK_{Ca}) channels. This K^+ current is known to act as a repolarization current. This large K^+ current is sensitive to TEA and can be eliminated by using Ca^{2+} channel blockers such as Cd^{2+}, nifedipine and verapamil (Terada, 1987; Imaizumi *et al.*, 1989). The other K^+ current found in guinea pig ureter has small conductance (I_{to}) which is not sensitive to TEA but can be abolished by 4-AP (Imaizumi *et al.*, 1989). I_{to} has properties similar to A-type current (I_a) observed in other types of smooth muscle and neurons. I_{to} can be activated and inactivated by changes in voltage. At the resting membrane potential, I_{to} is inactivated; its activation dependents on the K^+ gradient across the plasma membrane (Conor & Stevens, 1971; Adam *et al.*, 1980; Lang, 1989). However, unlike I_{KCa}, I_{to} does not act as a repolarizing current in action potential. In guinea pig ureter, inhibition of I_{to} with 4-AP increased the frequency and duration of the action potential. I_{to} plays a role in the after-hyperpolarization, the refractory period and the generation of the spontaneous action potential (Shuba, 1981; Exintaris *et al.*, 1999). It is suggested that I_{to} opposes I_{Ca} and may contribute to the rate of decay of the after-hyperpolarization (Imaizumi *et al.*, 1989; Okabe *et al.*, 1987; Beech & Bolton, 1988; Leech & Meech, 1988).

In rat ureteric smooth muscle, in addition to the two K^+ currents found in guinea pig

ureter mentioned above, a delayed inward rectifier type K^+ current (I_{Kr}) has also been observed (Smith *et al.*, 2002). I_{Kr} has been characterized well in many other excitable cells (Cooper *et al.*, 1991; Von *et al.*, 1996). In vascular smooth muscle cells, endothelin-1 and angiotensin II inhibit I_{Kr} by activating PKC and adenosine stimulates I_{Kr} by activating PKA (Park *et al.*, 2008). In the rat ureter, Cd^{2+} abolishes the inward current, STOCs and tail current but I_{Kr} remains especially after application of 4-AP at the end of depolarization pulse (Smith *et al.*, 2002).

2.2 Agonists and the action potential

By using the double sucrose-gap method, Shuba (1977 (1)) showed that agonists, such as adrenaline, noradrenaline, and histamine, increased the duration of the plateau component of the action potential which was accompanied by an increased duration and amplitude of force.

The agonists, such as noradrenaline or histamine can produce an increase in the intracellular Ca^{2+} concentration, either due to the release of Ca^{2+} from the intracellular store (SR) or the influx of Ca^{2+} from the extracellular space (Breemen *et al.*, 1989; Albert, 2003). Histamine depolarizes the cell membrane by affecting the passive ionic permeability of the membrane, and this is accompanied by a decreased membrane resistance. However, this can not explain the increased duration of the plateau component of the action potential.

It is suggested that the increased duration of the action potential by noradrenaline and histamine is due to the activation of Na^+ permeability. Shuba concluded that there is an effect on the potential-dependent conductance of the slow Na^+ channel which is responsible for the generation of the plateau component of the action potential (Shuba, 1977(1)). However, it has not been possible to detect any inward Na^+ current in ureteric smooth muscle until now. After blocking Ca^{2+} channels with a Ca^{2+} channel inhibitors such as nifedipine or elimination of extracellular Ca^{2+}, no inward current is detected. Therefore, it is suggested that there is an intracellular Ca^{2+} concentration dependent inward Na^+ current or an inward current entering via electrogenic Na^+ – Ca^{2+} exchange (Imaizumi *et al.*, 1989). Unfortunately, due to the limitations of the techniques used, the first possibility is unfit for examination.

2.3 Ion channels

2.3.1 The voltage-gated Ca^{2+} channel (VGCC)

The inward Ca^{2+} current displays different characteristics and kinetics in different smooth muscles. This may be accounted for by the different Ca^{2+} channels expressed in smooth muscle. Generally, several VGCCs are expressed in smooth muscle and have been reported to function in different types of smooth muscles- L-type, T-type and N-type voltage-gated Ca^{2+} channels (Jensen *et al.*, 2004; Salemme *et al.*, 2007). In ureteric smooth muscle, electrophysiological, pharmacological and immunohistochemical studies suggest that L-type Ca^{2+} channels are responsible for generating of the action potential that mediates excitation-contraction coupling (Lang,

1989; Imaizumi *et al.*, 1989; Santicioli & Maggi, 1998).

Depolarization and activation of Ca^{2+} entry via L-type Ca^{2+} channels is a fundamental mechanism in the physiological activation of ureteric smooth muscle as Ca^{2+} channel blockers nifedipine and verapamil suppress the inward Ca^{2+} current, action potential and phasic contractions (Shuba, 1977(2), 1980; Exintaris *et al.*, 1999; Lang *et al.*, 2006). It has been widely accepted that an inward Ca^{2+} current via L-type Ca^{2+} channel is the major depolarizing current to initiate action potential in smooth muscle (Bolton *et al.*, 1985; Ganitkevich *et al.*, 1986). At lower concentrations, nifedipine and verapamil can abolish the plateau component of the action potential, while a higher concentration of both drugs can completely abolish the generation of the action potential (Imaizumi *et al.*, 1989; Lang *et al.*, 1990; Smith *et al.*, 2002). Several characteristics of Ca^{2+} inward current have been identified in ureteric smooth muscle. In both rat and guinea pig ureter, the Ca^{2+} current can be completely abolished by the Ca^{2+} channel blockers nifedipine and verapamil, which results in a decreased duration and amplitude of action potential and eventually abolishment of the action potential (Lang, 1990; Smith *et al.*, 2002). High K^+ solution can fire an action potential by activating L-type Ca^{2+} channels. After application of a Ca^{2+} channel blocker, both the phasic and the tonic components of a contraction induced by elevation of extracellular K^+ concentration can be inhibited (Santicioli & Maggi, 1998; Sunano, 1976). Maggi *et al.* showed that PKA inhibitors can inhibit the tonic component of contraction induced by high K^+. PKA may play an important role in the modulation of L-type

Ca^{2+} channels and especially for the maintenance of the noninactivating state of the Ca^{2+} channels (Imaizumi *et al.*, 1989; Maggi *et al.*, 1996).

2.3.2 BK_{Ca} Channels

The BK_{Ca} channel is a large conductance voltage and intracellular Ca^{2+}-dependent channel. It has a uniquely large channel current and so is termed BK_{Ca} channel for its big K^+ current. Under voltage clamp conditions, the current through the BK_{Ca} channel appears as a spontaneous transient outward current (STOCs) first described by Bolton and Benham (Bolton & Benham, 1985).

The presence of STOCs in both guinea pig and rat ureteric smooth muscle cells has been demonstrated (Imiazumi *et al.*, 1989; Lang, 1989; Smith *et al.*, 2002; Borisoval *et al.*, 2007). The BK_{Ca} channel is normally activated by Ca^{2+} release from the SR in the form of Ca^{2+} sparks (Nelson *et al.*, 1995; Burdyga & Wray, 2005). The activity of K^+ outward current via BK_{Ca} channel has been shown to have the same kinetics as Ca^{2+} sparks (Nelson *et al.*, 1995; Bonev *et al.*, 1997; Jaggar *et al.*, 1998; Porter *et al.*, 1998). The activity of BK_{Ca} channels is increased by the increased Ca^{2+} spark activity caused by application of caffeine (Borisova *et al.*, 2007). In vascular smooth muscle, it has been shown that activation of PKC inhibits BK_{Ca} channel activity (Crozatier, 2006; Ko *et al.*, 2008). It has been suggested that in ureter smooth muscles BK_{Ca} channels serve distinct physiological functions such as control of the refractory period, excitability and the duration of the action potential (Burdyga & Wray, 2005; Borisova

et al., 2007).

2.3.3 Other K^+ channels

In addition to the BK_{Ca} channel, there are another two types of K^+ channel have been identified in ureteric smooth muscle: the voltage-dependant K^+ channel (K_v) and the delayed inward rectifier K^+ channel (K_{ir}) (Smith *et al.*, 2002).

The current through voltage-dependent K^+ channel has been found in both rat and guinea pig ureteric smooth muscle. The current has properties similar to the A-type current originally found in molluscan neurons (Connor & Stevens, 1971; Adam *et al.*, 1980). At a resting membrane potential of about –60mV, this channel is inactivated. However, these channels can be activated if voltage clamped ureteric myocites are depolarized to –30mV from a holding potential of –80 mV (Lang, 1989).

The delayed inward rectifier K^+ channel has been found in rat ureteric smooth muscle cells but not the guinea pig. It is insensitive to TEA or Ca^{2+} (Smith *et al.*, 2002). The delayed inward rectifier K^+ channel activity has been suggested to account for little effect of TEA on the duration of the action potential in rat ureter which is in contrast to its marked effect on the duration of the action potential in the guinea pig ureter (Smith *et al.*, 2002).

2.3.4 Cl_{Ca} Channel

Cl_{Ca} channels have been identified in rat ureteric smooth muscle by Smith *et al.* (Smith *et al.*, 2002). This channel is activated by Ca^{2+} and Cl^- efflux depolarizing the cell membrane which leads to opening of voltage-dependent Ca^{2+} channels and Ca^{2+} influx into the cells.

The Cl_{Ca} channel was first reported in amphibian tissue in rod segments from salamander retina. It has also been found in several smooth muscle tissues, vascular, airway, rat ureteric and uterine smooth muscle (Greenwood *et al.*, 1996; Arnaudeau *et al.*, 1997; Smith *et al.*, 2002). However, it is not present in all types of smooth muscle. For example, Cl_{Ca} channels are absent in guinea pig ureteric smooth muscle, which is thought to be one of the explanations for the species dependence mechanism of generation of the action potential in rat and guinea pig ureteric smooth muscle (Imaizumi *et al.*, 1989; Lang *et al.*, 1990).

Cl_{Ca} channels can be activated by excitatory neurotransmitters and other agonists. In the rat portal vein it was shown that noradrenaline can significantly increase Cl^- conductance and lead to membrane depolarization. Endothelin has been also shown to activate I_{ClCa} in vascular smooth muscle. Histamine can also increase Cl_{Ca} channel activity in airway smooth muscle (Large & Wang, 1996). The removal of extracellular Ca^{2+} eliminates I_{ClCa} suggesting that its activation is dependent on Ca^{2+} entry. In rat ureter, it is activated by Ca^{2+} influx via the L-type Ca^{2+} channel (Smith *et al.*, 2002).

Activation of the Cl_{Ca} channel can also be induced by Ca^{2+} release from the SR (Amedee *et al.*, 1990; Lamb *et al.*, 1994).

Activation of Cl_{Ca} channels produces spontaneous transient inward currents (STICs). STICs can be activated by local $[Ca^{2+}]_i$ increase such as Ca^{2+} sparks or Ca^{2+} puffs due to the opening of both IP_3 and RyR channels. Large I_{ClCa} can also be activated by Ca^{2+} waves (Mironneau *et al.*, 1996; Haddock *et al.*, 2002).

2.3.5 The Na^+-Ca^{2+} exchanger

The existence of the Na^+-Ca^{2+} exchanger in guinea pig ureteric smooth muscle has been demonstrated by Aickin, Brading and Burdyga (Aickin *et al.*, 1984). These authors demonstrated that it can operate in Na^+-loaded ureter. However, its function in ureteric excitation-contraction coupling remains to be demonstrated, although Aaronson and Benham showed the contribution of the Na^+-Ca^{2+} exchanger to Ca^{2+} flux in voltage clamped ureteric myocytes (Aaronson and Benham, 1989).

The Na^+-Ca^{2+} exchanger is electrogenic. Normally, it pumps out one Ca^{2+} ion in exchange for three Na^+ ions therefore generating a net inward current. The Na^+-Ca^{2+} exchanger is a reversible transporter and can operate in both "Ca^{2+} extrusion" or "Ca^{2+} entry" mode depending on the membrane potential and prevailing Na^+ and Ca^{2+} gradients.

So far, there is no specific Na^+-Ca^{2+} exchanger inhibitor found. Several studies show that manganese (Aickin *et al.*, 1987), La^{3+} (Weiss, 1974) and dodecylamine (Philipson *et al.*, 1985) all inhibit Na^+-Ca^{2+}exchanger activity in guinea pig ureteric smooth muscle. However, all of them have an additional effect on L-type Ca^{2+} channels (Aickin *et al.*, 1987). The lack of a specific inhibitor for the Na^+-Ca^{2+} exchanger accounts for the depth of studies into its contribution to the mechanism of smooth muscle contraction.

The Na^+-Ca^{2+} exchanger is not the only mechanism responsible for the regulation of Ca^{2+} homeostasis in smooth muscle by transporting Ca^{2+} out of the cell. An ATP-dependent Ca^{2+} pump is also found to extrude Ca^{2+} out of the cells to maintain the low intracellular Ca^{2+} concentration (Floyd & Wray, 2007).

2.4 Refractory period

The refractory period is a period of inexcitability during which the excitable tissue fails to respond to any stimulus (absolute refractory period) or requires a greater stimulus to generate a new action potential (relative refractory period). The existence of a refractory period is vital for setting the frequency of ureteric peristalsis and it plays an important role in preventing hyperactivity in excitable tissues (Santicioli & Maggi, 1998). It has been suggested that the refractory period of the spontaneous action potential is due to the opening of three K^+ channels- the Ca^{2+} activated K^+ channel, the voltage dependent Ca^{2+} insensitive K^+ channel and the K^+ inward

rectifier channel (Exintaris *et al.*, 1999).

Recently, the mechanism that underlies the refractory period in guinea pig ureteric smooth muscle has been clearly elucidated. This mechanism feeds back negatively to limit the excitability of the cells. The refractory period is mainly dependent on Ca^{2+} release from SR. In addition, inactivation of Ca^{2+} channels can regulate the myogenic component of the refractory period (Maggi *et al.*, 1994; Burdyga & Wray, 2005).

After firing the action potential, the membrane depolarizes, Ca^{2+} flows into the cells and the SR is filled with Ca^{2+}. As the SR Ca^{2+} content is increased, Ca^{2+} sparks are activated. This phase of excitability is followed by a subsequent phase of refractoriness. The increased Ca^{2+} sparks can activate plasmalemmal BK_{Ca} channels and produce a transient after-hyperpolarization and cause the membrane inexcitability. This is the fundamental basis for the mechanism of refractoriness (Figure 1.5). At the end of the refractory period, the Ca^{2+} spark activity becomes the lowest (Burdyga & Wray, 2005). Therefore, BK_{Ca} channel activity is decreased. After the refractory period is terminated, the stimulus can produce a new action potential (Wray *et al.*, 2005).

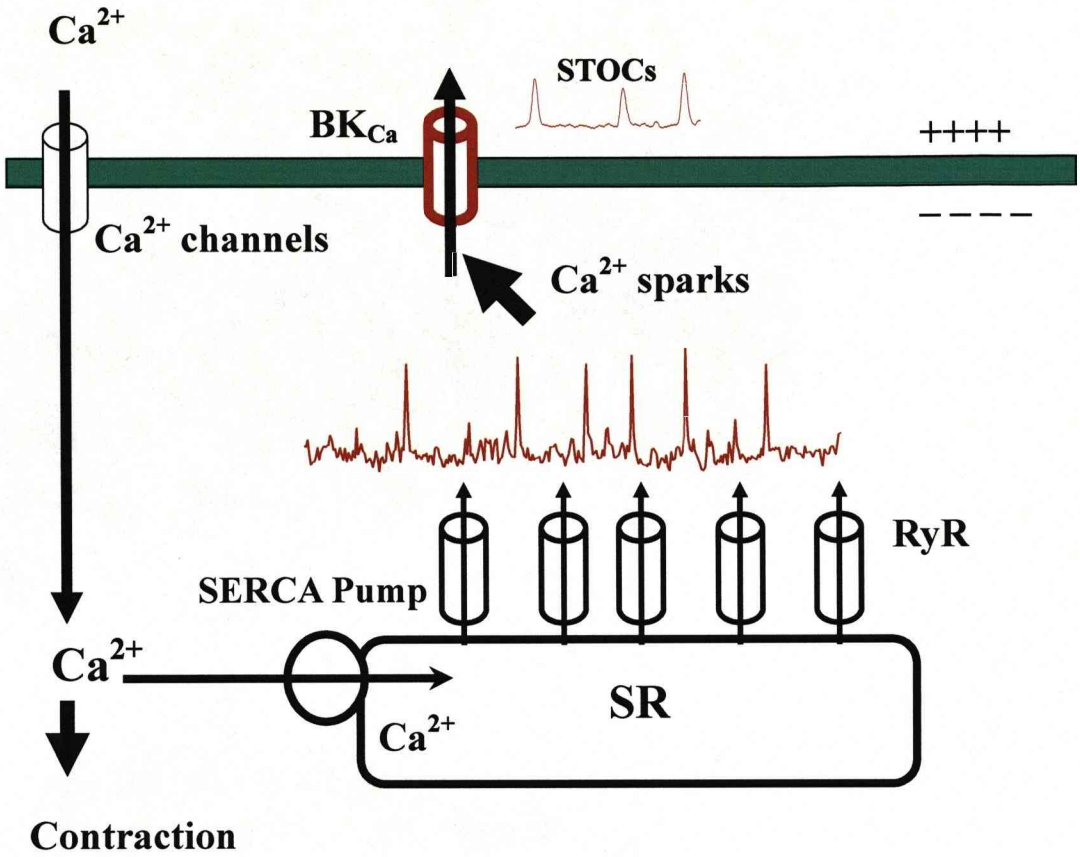


Figure 1.5 Diagram illustrating molecular mechanism of refractory period in guinea pig ureter smooth muscle cells. Refractory period is mainly dependent on Ca^{2+} release from SR. Ca^{2+} entry into SR via Ca^{2+} pump to generate Ca^{2+} sparks which activate nearby BK_{Ca} channels leading to membrane hyperpolarization.

3 The mechanism of activation of smooth muscle contraction

Activation of smooth muscle contraction is triggered by a rise in intracellular Ca^{2+} either due to Ca^{2+} entry into the cell via L-type voltage-gated Ca^{2+} channels or Ca^{2+} release from intracellular SR store via IP_3 receptors or ryanodine receptors. After the intracellular Ca^{2+} concentration has increased, there is more free Ca^{2+} in cytoplasm that can bind to calmodulin. Each calmodulin molecule can bind up four Ca^{2+} ions and cause a conformational change that results in exposure of the hydrophobic sites responsible for binding of myosin light chain kinase(MLCK) (Allen *et al.*, 1994; Walsh, 1994). After this activation of calmodulin binding to the catalytic subunit of MLCK, it forms an active Ca^{2+}/Calmodulin/MLCK complex that phosphorylates the Ser19 of two myosin light chains and initiates the contraction.

3.1 Contractile proteins

Each smooth muscle cell contains thick filaments (mysosin) and thin filaments (actin). Smooth muscle contraction results from the formation of cycling cross-bridges between actin and myosin filaments. Skeletal muscle and smooth muscle share some muscle proteins including actin, myosin and tropomyosin. However, smooth muscle possesses specific isoforms of several proteins in contrast to skeletal muscle, including caldesmon, calponin and calmodulin. These proteins are abundant in smooth muscle and play an important role in its contraction (Rasmussen *et al.*, 1987).

3.1.1 Myosin

Myosin is a family of motor proteins that can move along actin. Myosin II has been found in smooth muscle and is responsible for smooth muscle contraction. Each myosin molecule is composed of two heavy chains (230kDa) and two pairs of light chains (20kDa and 17kDa each). Each myosin molecule contains two globular heads and a long, rod-like tail. The heads contain actin binding sites and ATP hydrolysis sites. Myosin light chain lies in the head part. The tail part contains two myosin heavy chains (Craig *et al.*, 1983; Walsh, 1994). Myosin light chains have to be phosphorylated for binding actin (Kohama *et al.*, 1996; Hatch *et al.*, 2001). The movement of myosin along actin is ATP-dependent and is accompanied by ATP hydrolysis.

3.1.2 Actin

Thin-filament is chiefly composed of actin and a small amount of some other regulated proteins. Therefore, actin is often referred to as thin filament. Actin is a globular and about 42 kDa protein.

Thin filament may contribute to the regulation of "latch state" in smooth muscle. The thin filaments are capable of strong-binding or rigor-like cross bridge to "turn-on" the actin filament. The activated actin filament is also able to activate the unphosphorylated smooth muscle myosin in some types of smooth muscles and stimulate the formation of slowly cycling cross-bridges in the absence of calcium

(Haeberle, 1999). Regulation of thin filament is essential for smooth muscle contraction. This can be achieved by modulation of its binding protein, such as calponin and caldesmon.

3.1.3 Calponin

Calponin is a homologous actin filament-associated protein of 292-330 amino acids expressed in both smooth muscle and non-muscle cells. Calponin binds to actin in a Ca^{2+}-independent manner. The binding of calponin to actin inhibits the myosin MgATPase without affecting myosin phosphorylation. Calponin can be phosphorylated either by PKC or Calmodulin-dependent protein kinase II (CaM kinase II) and this leads to calponin-mediated inhibition of the myosin MgATPase (Winder *et al.,* 1993).

3.1.4 Caldesmon

Same as calponin, caldesmon has been found in both smooth muscle and non-muscle cells. Caldesmon binds to actin to inhibit activation of myosin MgATPase activity. Therefore, caldesmon acts as an inhibitory mechanism to prevent smooth muscle uncontrolled contraction. The inhibitory effects of caldesmon can be reversed by binding of Ca^{2+}/Calmodulin.

3.1.5 Calmodulin

Calmoduin is an important protein playing a key role in control of smooth muscle

contraction. It is expressed in other types of muscle tissues, such as striated muscle, cardiac muscle which contraction is achieved mainly by the troponin/tropomyosin complex associated with the actin filaments (Weber & Murray, 1973). Smooth muscle does not possess troponin, but its contraction depends on calmodulin, which upon binding Ca^{2+} activates MLCK and phosphorylates myosin light chains to activate cross bridge cycling (Kohama *et al.*, 1996).

Calmodulin is a Ca^{2+} binding protein. In addition to Ca^{2+}, it can also bind various proteins including: caldesmon, calponin and CaM kinase II. These calmodulin binding proteins also contribute to the smooth muscle contraction. CaM kinase II contains three major domains: an N-terminal regulatory domain containing a Ser/Thr kinase site and Ca^{2+}/Calmodulin binding site, a central-linker domain, and a C-terminal association domain. Activation of CaM kinase II results from Ca^{2+}/Calmodulin binding to its regulatory domain and increases its affinity for Ca^{2+}/Calmodulin (William *et al.*, 2005). In turn, the activated CaM kinase II phosphorylates myosin light chain (Walsh *et al.*, 1994).

Calmodulin has four Ca^{2+} binding sites, namely I, II, III and IV. Each binding site can bind one Ca^{2+} ion. After first two Ca^{2+} ions bind to calmodulin, a conformational change occurres. The hydrophobic sites of calmodulin are exposed which results in its binding to MLCK (Laporte *et al.*, 1980). Occupation of at least three Ca^{2+} binding sites by Ca^{2+} is needed to activate the MLCK activity (Blumenthal *et al.*, 1980).

3.2 Mechanisms of regulation of Ca^{2+}-sensitivity of contraction in smooth muscle

The relationship between myosin phosphorylation and force development is not linear (Rembold, 1992). The force can be maintained at a low phosphorylation level. It occurs with a decline in $[Ca^{2+}]_i$ from its peak level but still higher than resting $[Ca^{2+}]_i$ and a decreased activity of MLCK and low phosphorylation level of myosin light chain with a sustained force and reduced rates of cross-bridge cycling and ATP hydrolysis. This process is referred to as latch state which allows smooth muscle contraction with little energy expenditure (Dillon & Murphy, 1982; Harberle, 1999).

When the cytoplasmic Ca^{2+} concentration is restored to its resting levels, the relaxation of smooth muscle occurs. Calmodulin is dissociated from the Ca^{2+} and the Ca^{2+}/Calmodulin/MLCK complex become inactive which results in a further decrease in MLCK activity. Myosin light chain phosphatase (MLCP) is Ca^{2+}/Calmodulin independent, dephosphorylates myosin light chain which leads to relaxation (Haeberle *et al.*, 1985; Himpens *et al.*, 1988).

Phosphorylation of the 20kDa myosin light chain (MLC_{20}) facilitates actin myosin binding and initiates cross-bridge cycling. In general, MLC_{20} phosphorylation can be controlled by either Ca^{2+}-dependent mechasnisms via activation of Ca^{2+}/Calmodulin dependent MLCK or by Ca^{2+}-independent mechanisms via regulation of MLCP activity by some kinases, including Rho-kinase and protein kinase C (PKC) (Kamm *et*

al., 2001). MLCP can dephophorylate myosin light chain and lead to inactivation of actin-activated ATPase. Thus, phosphorylation of MLC_{20} can be modulated by both myosin light chain phosphatase and myosin light chain kinase.

3.2.1 MLCK

MLCK is an enzyme that catalyzes the phosphorylation of the myosin light chain (Herring *et al.*, 2000). It contains a kinase catalytic subunit, a calmodulin binding site, an autoinhibitory site, and several structural motifs (Allen *et al.*, 1994; He *et al.*, 2008). MLCK phosphorylates S19 of the MLC_{20}. At higher concentration, it also phosphorylates at T18 of myosin light chain (Ikebe *et al.*, 1986). MLCK can bind to actin and myosin. MLCK activity is Ca^{2+}/Calmodulin dependent.

At the resting state of smooth muscle, MLCK activity is inactive which is due to myosin-binding site being occupied by a pseudosubstrate domain (Walsh, 1994). Upon stimulation, the MLCK is activated by Ca^{2+}/Calmodulin complex and the pseudosubstrate domain is removed therefore allowing myosin binding to MLCK.

3.2.2 MLCP

MLCP can dephosphorylate MLC_{20}. It contains 3 subunits: a large 110-130 kDa regulatory subunit which binds myosin (MYPT1), a 37-38 kDa catalytic subunit (PP1C) and a small 20kDa subunit whose function is unknown. Phosphorylation of the MYPT1 and the catalytic subunit reduces MLCP activity (Hartshorne *et al.*, 1998;

Shimizu *et al.*, 1994).

Inhibition of MLCP occurs in response to various agonists (Deng *et al.*, 2002). Two ways has been identified in which the MLCP activity can be inhibited- one way is Rho kinase through receptor coupled to G proteins (Somlyo *et al.*, 2000); the other way is PKC action through receptor coupling with $G_{q/11}$ and PLC (Kitazawa *et al.*, 2000).

3.2.3 Ca^{2+} sensitization

Ca^{2+} sensitization of smooth muscle reflects a ratio of activities of MLCK to MLCP. The level of phosphorylation of myosin light chain can be modulated by pushing the equilibrium between MLCP and MLCK.

Phosphorylation level of [MLC_{20}] = function of (MLCK activity-MLCP activity)

Both increased MLCK activity or decreased MLCP activity produces an increase in MLC_{20} phosphorylation level which leads to activation of cross-bridge cycling and contraction of smooth muscle at a constant level of cytoplasmic free Ca^{2+} concentration. Conversely, decreased MLCK activity or increased MLCP activity leads to decreased MLC_{20} phosphorylation and inactivation of actin-activated myosin ATPase. When Ca^{2+} desensitization occurs there is a decline of force and MLC_{20} phosphorylation at the constant level of the intracellular $[Ca^{2+}]_i$ (Himpens *et al.*, 1989).

The Ca^{2+} sensitization mechanism in smooth muscle involves inhibition of MLCP by agonist binding ligand receptors, G proteins, and guanine nucleotide-binding factors (Somlyo *et al.*, 2003). This can be regulated by either Rho A/Rho Kinase mechanism or CPI-17/PKC action which is discussed in detail below.

3.2.4 Agonist-induced contraction

In smooth muscle, binding of an agonist to its receptor on the cell membrane induces a series of signal transduction steps which eventually initiate smooth muscle contraction. In addition, the sensitivity of the contractile apparatus to Ca^{2+} can be regulated by agonists. In smooth muscle, two mechanisms have been found so far which is involved in regulation of agonist induced Ca^{2+} sensitization:

(1) Agonists (such as cabachol, oxytocin) can bind to G-Proteins of the $G_{q/11}$ family on plasmalemma membrane which in turn activates phospholipase C (PLC). PLC can mediate hydrolysis of PIP2 into inositol 1.4.5-trisphosphate (IP_3) and 1,2-diacylglyceral (DAG) respectively. IP_3 binds to its receptor on the SR surface and causes Ca^{2+} release from SR. DAG activates PKC (Walsh, 2007). PKC in turn phophorylates CPI-17 at Thr38 and enhances its inhibitory potency of MLCP. The myosin light chain kinase/myosin light chain phosphatase ratio is moving towards MLCK, resulting in an increase in myosin light chain phosphorylation (Figure 1.6).

(2) Agonists can also bind to $G_{\alpha q}$ / $G_{\alpha 12,13}$ coupled receptors to activate RhoA and its downstream effectors. In the resting state, the inactivated form of RhoA is bound to RhoGDI (guanine nucleotide dissociation inhibitor). The RhoA/RhoGDI complex can

be activated by Rho-GEFs (guanine nucleotide exchange factors) to exchange GDP for GTP on RhoA and the activated RhoA is dissociated from the complex and translocates to the plasma membrane and in turn activates the Rho-kinase. The activated Rho-kinase phophorylates the regulatory subunit of MLCP and therefore inhibits MLCP activity (Cherfils & Chardin, 1999; Somlyo *et al.*, 2000).

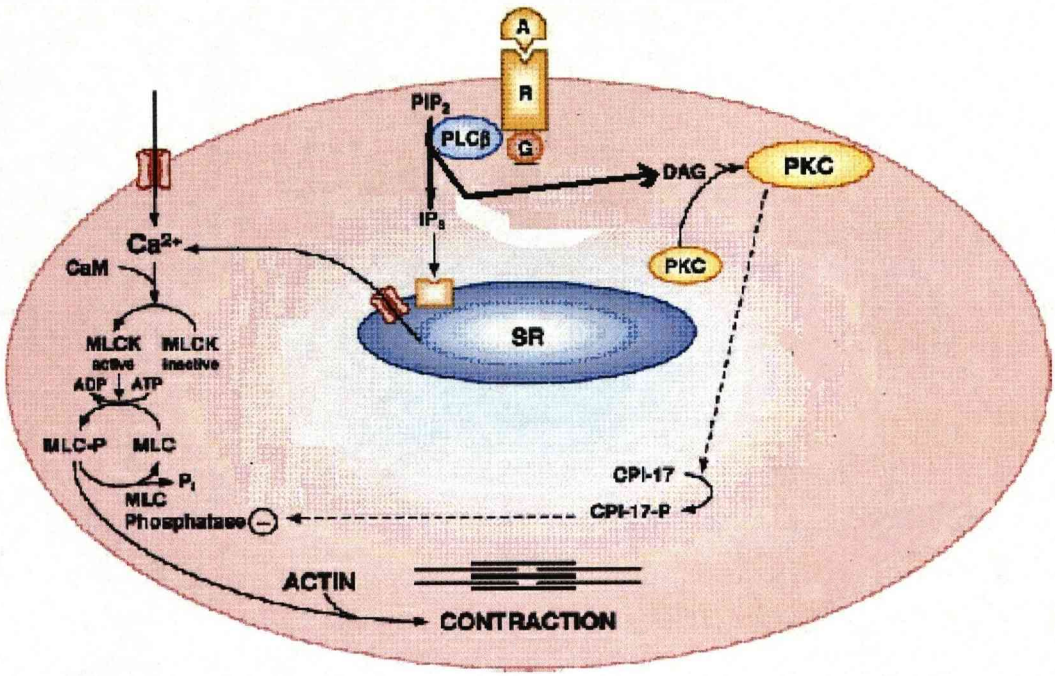


Figure 1.6 Diagram showing possible mechanism of agonist-induced smooth muscle contraction. Agonist (A) binds to its receptor (R), activates plasma membrane PLC, and increases production of IP_3 and DAG. IP_3 stimulates Ca^{2+} release from the SR. DAG activates PKC. PKC phosphorylates CPI-17, which in turn inhibits MLC phosphatase and thereby increasing the myosin light chain phosphorylation level and force in Ca^{2+}-independent way. (Figure modified from Salamanca, D. A. and Khalil, R A. 1995).

3.2.5 RhoA/ROCK pathway

RhoA is a small about 20 kDa monomeric GTPase protein. RhoA can be activated when it is bound to GTP (referred to Rho GTP) and is inactivated when it is bound to GDP. The activated RhoGTP in turn stimulates Rho-kinase, which is serine/threonine kinase and it plays an important role in various cellular functions. Rho-kinase contains a Rho-binding domain and can be activated by RhoGTP (Leung *et al.* 1995; Matsui *et al.* 1996). The process of activation of Rho-kinase requires RhoA GTP translocation to the plasma membrane (Gong *et al.* 1997; Taggart *et al.* 1999). In addition, Rho-kinase can be activated by arachidonic acid and contributes to Rho-kinase mediated Ca^{2+} sensitization (Fu *et al.*, 1998; Feng *et al.*, 1999). Rho-kinase phosphorylates MLCP 130kDa myosin binding sites and therefore inhibits MLCP activity (Kimura *et al.*, 1996) resulting in enhancement of MLC_{20} phosphorylation and smooth muscle contraction at a constant level of $[Ca^{2+}]_i$.

Rho-kinase has been shown to play an important physiological role in modulation of smooth muscle contraction in a variety of smooth muscles. In tonic smooth muscle, Y-27632 (Rho-kinase inhibitor) can reduce blood pressure without affecting normal blood pressure (Uehata *et al.*, 1997). In ureter smooth msucle, Rho-kinase has been shown to be involved in the excitation-contraction coupling in two ways-Ca^{2+} dependant and Ca^{2+} independent mechanism (Shabir *et al.*, 2004). The inhibition of Rho-kinase affects the duration of action potential, Ca^{2+} transient and force by targeting the L-type voltage-gated Ca^{2+} channels (Ca^{2+}-dependant pathway) (Shabir *et*

al., 2004). In addition, Rho-kinase was also found to modulate the ureter smooth muscle contraction in a Ca^{2+}-independent way by modulating MLCP activity. The effect of Rho-kinase on ureter smooth muscle is species dependent which is only shown in rat but absent in guinea pig ureter smooth muscle (Shabir *et al.*, 2004).

3.2.6 CPI-17 and PKC

CPI-17 is 17 kDa peptide. It can inhibit type 1 serine/threonine phosphatase, such as MLCP. Phosphorylation of CPI-17 at Thr38 by PKC increases its inhibitory potency about 1000 fold. In addition to PKC, some other kinases can also phosphrylate the Thr38 site of CPI-17, such as ROCK, PKN and PKA (Koyama *et al.*, 2000; Hamaguchi *et al.*, 2000; Takizawa *et al.*, 2002). CPI-17 inhibits the catalytic subunit of MLCP, which provides an independent pathway for MLCP inhibition without affecting its myosin binding subunit (Li *et al.*, 1998; Kitazawa *et al.*, 2000).

Inhibition of Rho-kinase with Y-27632 and PKC with GF109203X reduced CPI-17 phosphorylation and decreased contraction suggest that Rho-kinases and PKC are downstream of CPI-17 (Kitazawa *et al.*, 2000, 2003).

Some studies show that activation of conventional or novel PKC by phorbol esters and diacylglycerol (DAG) can inhibit the activity of MLCP (Ioh *et al.*, 1993; Masuo *et al.*, 1994). The PKC activator induced Ca^{2+} sensitization independently of changes of $[Ca^{2+}]_i$ and can not be inhibited by Y-27632 (Fu *et al.*, 1998). It suggests that PKC

activation induces Ca^{2+} sensitization by inhibiting MLCP activity but it is not through the RhoA/ROCK pathway. PKC physiologically modulate Ca^{2+} sensitization by phosphorylation of CPI-17.

4 Ca^{2+} signaling in ureter smooth muscle

4.1 Role of extracellular calcium

At rest, $[Ca^{2+}]_i$ in majority of smooth muscle cells is 0.1-0.2 μM. Upon stimulation, $[Ca^{2+}]_i$ can rise up to 0.5-0.7 μM either due to Ca^{2+} entry via voltage-gated or receptor-operated Ca^{2+} channels or Ca^{2+} release from sarcoplasmic reticulum (SR) (William & Fay, 1986; Allen & Walsh, 1994). So far, it is known that Ca^{2+} entry into the cell provides a major triggering source to initiate smooth muscle contraction. In ureter smooth muscle, Ca^{2+} entry via L-type Ca^{2+} channels plays a key role in control of phasic contraction (Carlo *et al.*, 1995). $[Ca^{2+}]_i$ is maintained by a number of mechanisms which include Ca^{2+} extrusion from cell by a Ca^{2+} pump in the plasma membrane and the Na^+-Ca^{2+} exchanger. Ca^{2+} can also be sequestered into intracellular store by SERCA pump on SR membrane.

4.2 Role of the SR

In addition to Ca^{2+} entry from the extracellular fluid, Ca^{2+} release from the intercellular store can also play an important role in control of the Ca^{2+} signaling in ureter smooth muscle.

The SR functions as an internal Ca^{2+} store uptaking and releasing Ca^{2+} from the store. Ca^{2+} can be taken into the SR by SERCA pump from the cytoplasm by expense of ATP. Generally, in smooth muscle, two types of intracellular Ca^{2+} release channels have been characterized: Ryanodine receptor (RyR) and Inositol 1,4,5-trisphosphate

(IP_3) receptors. They are responsible for removing the Ca^{2+} from the internal store.

Role of the SR function in smooth muscles is still poorly understood. In tonic smooth muscle, Ca^{2+} release during agonist stimulation can contribute to generation of vascular tone via generation of Ca^{2+} oscillations which appear in the form of propagating Ca^{2+} waves (Wray *et al.*, 2005). Both IP_3R and RyR channels have been reported to be involved in generation of Ca^{2+} oscillations. In phasic smooth muscle, such as bladder, activation of RyR channels can be triggered by Ca^{2+} entering the cell via L-type Ca^{2+} channels so called Ca^{2+}-induced Ca^{2+} release (CICR) and thus acts as an amplifying mechanism. In ureter smooth muscle, CICR is confined to microdomain and appear as localized Ca^{2+} sparks which act as a negative feedback mechanism to control ureteric excitability (Burdyga & Wray, 2005).

4.3 Ca^{2+} sparks

Ca^{2+} spark is a small local Ca^{2+} release event from clusters of RyR on the SR. The area where the Ca^{2+} is discharged is called frequent discharge site. In guinea pig ureter, this site is present and enriched with RyRs (Wray *et al.*, 2005). Smooth muscle cells have different isoforms of RyR subtypes RyR1, RyR2, and RyR3, but it is unclear that which isoform is necessary for Ca^{2+} sparks activity in ureter smooth muscle.

Ca^{2+} release via RyR channels can be activated by Ca^{2+} entering the cell through the voltage-dependent Ca^{2+} channels by activation of CICR mechanism. Caffeine can

facilitate CICR by sensitizing RyR to Ca^{2+}. In guinea pig ureter smooth muscle, caffeine can activate Ca^{2+} release from the SR producing an inhibition of action potential, phasic contraction and Ca^{2+} transient (Borisova *et al.*, 2007). However, it is not observed in rat ureter smooth muscle. Furthermore, Ca^{2+} sparks can be detected in guinea pig ureter but not in rat (Burdyga, *et al.*, 1998). These differences can be explained by the different IP_3 and RyR expression in the two species. Rat ureter was reported to express mainly IP_3R (Boiffin *et al.*, 2000; Burdyga *et al.*, 1995, 1998).

4.4 Ca^{2+} sparks/STOCs coupling mechanism in ureter smooth muscle

Ca^{2+} sparks can activate BK_{Ca} channel on the membrane to generate STOCs which is found in both guinea pig and rat ureter smooth muscle and is dominant in guinea pig. This Ca^{2+} sparks/STOCs coupling mechanism plays an important role in control of excitability in ureter smooth muscle.

Loading the SR store with Ca^{2+} facilities generation of Ca^{2+} sparks. Ca^{2+} sparks activate BK_{Ca} channels to produce STOCs and lead to membrane hyperpolarization and decrease the tissue excitability. Ca^{2+} sparks/STOCs coupling mechanism acts as a negative feedback mechanism to limit the cell membrane excitability (Figure 1.7).

Regulation of Ca^{2+} sparks/ STOCs coupling mechanism can affect not only the membrane excitability but also the duration of plateau component of the action potential, Ca^{2+} transient and phasic contraction in guinea pig ureter smooth muscle.

Activation of Ca^{2+} sparks by low concentration of caffeine increases the amplitude, frequency and kinetics of Ca^{2+} sparks and STOCs leading to hyperpolarization of the guinea pig ureter smooth muscle cells which results in an inhibition of the contractile activity. This effect of caffeine can be reversed by ryanodine or CPA or BK_{Ca} channel blocker TEA (Borisova *et al.*, 2007).

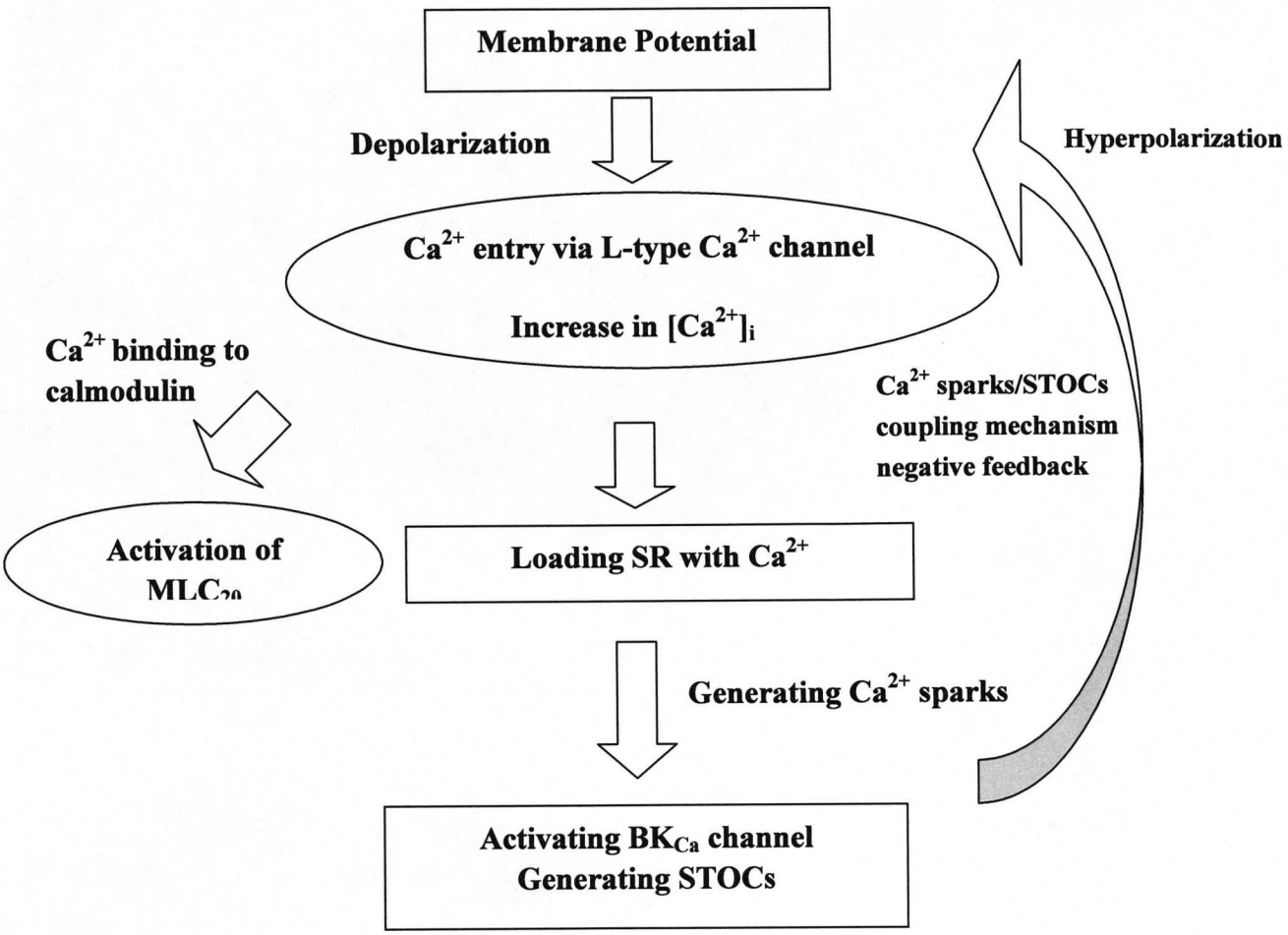


Figure 1.7 Ca^{2+} sparks /STOCs coupling mechanism in the guinea pig ureter smooth muscle. Depolarization of membrane opens the L-type Ca^{2+} channel results in an increase in intracellular Ca^{2+} leading to Ca^{2+} loading into SR. Increase in luminal $[Ca^{2+}]_i$ activates spontaneous Ca^{2+} sparks which could hyperpolarize the membrane by activating STOCs via Ca^{2+} sparks/STOCs coupling mechanism acting as a negative feedback mechanism to decrease cell excitability and duration of the plateau component of action potential.

4.5 Ca^{2+} puffs

There are three isoforms of IP_3 receptors, referred to IP_3R1, IP_3R2 and IP_3R3. The opening of clusters of IP_3 receptors can induce the spontaneous transient local Ca^{2+} release events, named Ca^{2+} puffs. The mechanism of Ca^{2+} release from IP_3 receptors is referred as IP_3-induced Ca^{2+} release (IICR). Agonist induced Ca^{2+} release from the SR can be activated in rat ureter smooth muscle. In rat ureter, IICR targets Cl_{Ca} channels and depolarizes the cell membrane and increases the Ca^{2+} entry into the cell via L-type Ca^{2+} channel (Burdyga *et al.*, 2002). Carbachol can evoke a contraction which is insensitive to ryanodine but can be blocked by heparin and cyclopiazonic acid in rat ureter, therefore, it is suggested that IP_3 expresses exclusively in rat ureter. (Burdyga *et al.,* 1998).

In some types of smooth muscles, both IP_3 receptor and ryonadine receptor simultaneously localized at the surface of SR store and interact with each other. Inhibition of IP_3 decreased frequency of Ca^{2+} sparks in rabbit portal vein myocites. In contrast, inhibition of IP_3 increased the frequency of Ca^{2+} sparks in guinea pig vas deferens smooth muscle cells (White *et al.*, 2003).

4.6 Ca^{2+} waves

The small localized Ca^{2+} release, Ca^{2+} sparks and Ca^{2+} puffs, can develop into Ca^{2+} waves (Wray *et al.*, 2005). The Ca^{2+} transient observed in intact smooth muscle preparation, propagating within individual muscle cells is also referred to as a Ca^{2+}

wave. Ca^{2+} release from SR provides an elementary events leading to Ca^{2+} waves which is caused either due to the intracellular Ca^{2+} release via IP_3 receptor or via RyR. In addition, agonists can induce smooth muscle contraction by initiating or amplifying Ca^{2+} wave activity by activation of PLC (McCarron *et al.*, 2002).

5 Smooth muscle and PKC

5.1 Smooth muscle

Generally, there are three types of muscles: smooth muscle, skeletal muscle and cardiac muscle. Smooth muscle is so called because of its distinguished characteristic lack of visible cross striations. Smooth muscle is responsible for the contractility of hollow organs, such as ureter, blood vessels, uterus and the airway. It maintains homeostasis and adaptive responses to stresses (He *et al.*, 2008). The impaired contraction of smooth muscle underlies several diseases including hypertension and bronchial asthma (Ihara *et al.*, 2007).

There are two types of smooth muscle in the body: multi-unit and visceral smooth muscle. Most of the internal hollow organs are visceral muscle which can be divided into phasic smooth muscle (ureter and uterus) and tonic smooth muscle (stomach fundus) based on the characteristics of contractile patterns and whether the spontaneous activity is present. The phasic smooth muscle like ureter and uterus contract and relax rapidly. The tonic smooth muscle contract slowly and may maintain the contraction for a prolonged periods.

5.2 PKC isoform and structure

Protein kinase C (PKC) is a family of serine threonine kinases. PKC family is divided into 3 groups: conventional, novel and atypical. Conventional PKC (cPKC) includes α, β and γ isoform. It requires Ca^{2+}, DAG, a phospholipid such as phosphatidylcholine

for activation. Novel PKC (nPKC) includes δ, ε and θ isoform. It requires DAG, but do not require Ca^{2+} for activation. Thus, conventional and novel protein kinases C are activated through the same signal transduction pathway as phospholipase C. Atypical protein kinases C (aPKC) include ξ and λ/ι isoform. Their activation are not dependent on Ca^{2+} or DAG.

Generally, PKC family consists of a catalytic domain and a regulatory domain. The regulatory domain is the site binding to phorbol/DAG (C1) or Ca^{2+} (C2). While the catalytic domain contain ATP (C3) and a substrate binding site(C4). The conventianal PKC has C1-C4 domain; the novel PKC isoform do not have the C2 region therefore do not require Ca^{2+} for activation.; the atypical PKC has only part of the C1 region and lack of C2 region therefore it is not affected by phorbol esters, DAG and Ca^{2+}(Salamanca *et al.*, 2005) (Figure 1.8).

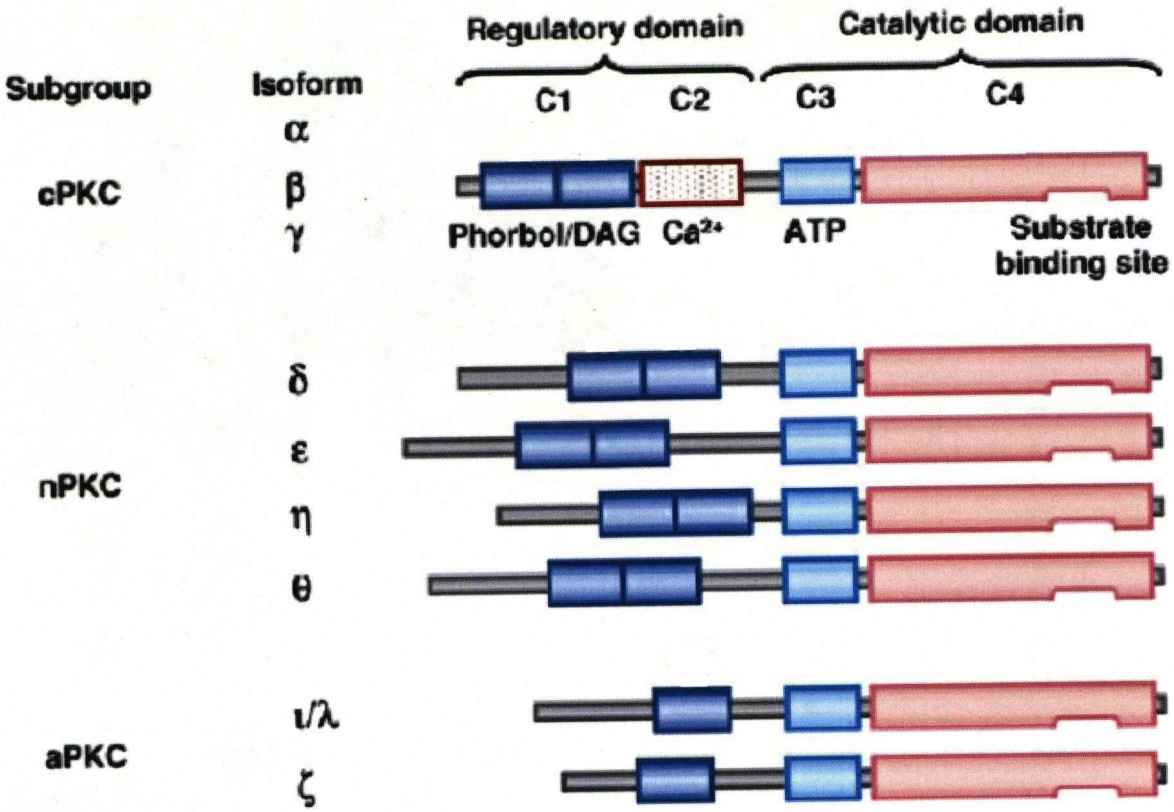


Figure 1.8 Structure of PKC isoforms. PKC consists of regulatory domain (C1 and C2) and catalytic domain (C3 and C4). (Figure modified from Salamanca, D. A. and Khalil, R A. 1995).

5.3 Signal pathways involving PKC in smooth muscle

Stimulation of smooth muscles by GPCR by agonists leads to hydrolysis of PIP2 and subsequent production of second messengers IP_3 and DAG.. DAG activates PKC and results in its translocation from the cytosol to the plasma membrane. Activation of PKC in turn induces a series of signal transduction in smooth muscle contraction including modulation of MLCP via CPI-17 and thereby affecting MLC_{20} phosphorylation.

PKC can be activated by the fatty acid derivative DAG, with Ca^{2+} acting as a cofactor for some isoforms. PKC can bind to Ca^{2+} in a phospholipid-dependent mechanism in addition to DAG. Calcium exerts its effect as a bridge between PKC and phospholipids (Bazzi *et al.*, 1990). PKC activator includes Phorbol esters, such as TPA, PMA and PDBu, which are the substrates for DAG in PKC activation. PKC inhibitors acting on the catalytic domain such as H-7 and Staurosporine are not specific. However, PKC inhibitors acting on the regulatory domain and competing at the DAG/ phorbol ester or the phosphatidylserine-binding site are more specific. This kind of inhibitors includes Calphostin C and Sphingosine (Salamanca *et al.*, 2005). However, the molecular effect of PKC specific inhibitor GF109203X and Ro320432 on PKC region is not clear. Ro320432 has been shown to inhibit specifically PKC isoforms α, βI, βII, γ and ε. GF109203X has specific inhibitory effect on isoforms α, βI, δ, ε, and ζ. Rottlerin has been shown to be a specific PKCδ inhibitor.

5.4 Function of PKC in smooth muscle

5.4.1 Ca^{2+} sensitization

PKC has been demonstrated to play a crucial role in regulation of several types of smooth muscle contraction, i.e. vascular, gut and airway smooth muscle (Murthy, 2005). In tonic smooth muscle such as arterial smooth muscle, activation of PKC enhances the force without or little change of intracellular free Ca^{2+} concentration by raising the sensitivity of the myofilaments to intracellular free Ca^{2+} (Rasmussen *et al.*, 1984, Chatterjee & Tejeda, 1986; Ruegg, 1999). This mechanism has been demonstrated recently to be achieved by inhibition of MLCP through mediation of a novel RACK- CPI-17 (Kitazawa *et al.,* 1999).

Agonists such as histamine or KCl can enhance the force which is associated with increased PKC activity (Singer *et al.*, 1992). In bovine and human airway smooth muscle, PKC is involved in the histamine induced contraction (Yang & Black, 1995; Boterman *et al.*, 2005). It suggests that agonists such as histamine activate PLC, producing an increase of IP_3 and DAG production and therefore activation of PKC (Boterman *et al.*, 2005).

5.4.2 PKC and ion channels

PKC has effect on plasma membrane channels and therefore membrane excitability. Activation of PKC is shown to inhibit BK_{Ca} channel activity in pulmonary vascular smooth muscle (Barnab *et al.*, 2004; Crozatier *et al.*, 2006; Ledoux *et al.*, 2006). The

agonists endothelin-1 and angiotensin II inhibit K_{ir} channel activity by activating PKC (Park *et al.*, 2008). PKC can also exert its effect on Ca^{2+} release from SR therefore affecting the activity of target channels and smooth muscle contraction. In vascular smooth muscle, activation of PKC decreases Ca^{2+} spark activity and therefore decreases STOCs frequency and hence modulation of membrane potential (Bonev *et al.*, 1997). In mouse airway smooth muscle cells, PKC activator inhibits Ca^{2+} sparks activity. Inhibition of PKC activates Ca^{2+} sparks frequency, which is due to the modulation through PLC and DAG (Liu *et al.*, 2007). Furthermore, in rabbit portal vein smooth muscle cells, the enhanced L-type Ca^{2+} channel activity is associated with activation of PKC (Ding *et al.*, 2004).

To our knowledge, nothing is known whether PKC is expressed and functionally important in phasic ureter smooth muscle. Therefore, in this study we have investigated possible role of PKC in control of ureteric contractility.

Hypothesis and Aims:

Hypothesis:

In many types of smooth muscle, e.g., vascular and bladder, PKC is involved in control of smooth muscle contraction. Therefore, we hypothesized that PKC is important in the regulation of excitation-contraction coupling mechanism in guinea pig and rat ureter smooth muscle.

Aims:

I. Using western blotting and immunohistochemistry to investigate the expression and distribution of different PKC isoforms in the guinea pig and rat ureter.

II. To study the effects of direct PKC activation and inhibition on the excitation-contraction coupling in the guinea pig and rat ureter smooth muscle by using different experimental models and protocols which include:

1. Investigation of the effects of PKC activator PDBU and PKC inhibitor Ro320432 on the temporal relationship between Ca^{2+} transients and force for phasic contraction evoked by electrical field stimulation in both guinea pig and rat ureter smooth muscle;

2. Elucidation of role of Ca^{2+} sparks/STOCs coupling mechanism and Na^{+}-Ca^{2+} exchanger in the effects of PKC activation on force-Ca^{2+} relationship in the ureter smooth muscle;

3. Investigation of functional role of Ca^{2+} sensitization in modulation of contractile activity by PKC activation in ureteric smooth muscle by using different experimental models and protocols;

III. To investigate possible role of PKC in the stimulant effects of agonists in the ureteric smooth muscle.

Chapter 2

Materials and Methods

Chapter 2 Materials and Methods

2.1 Animals

The ureters were obtained from male guinea pigs (300-400g) and male or female Wistar rats (250g). Animals were humanely killed by exposure to CO_2 in accordance with Animals (scientific procedures) Act of 1986. This was followed by cervical dislocation. The ureters were dissected and cleared of any connective tissues and fat and cut into small strips around 3-4mm in length under a dissection microscope. All ureters were immersed in buffered physiological Krebs solution (see 2.2) after isolation from the animal.

2.2 Solutions and chemicals

All chemicals and solutions were obtained from Sigma (Dorset, UK) unless otherwise stated.

In the experiments measuring force and calcium, the ureteric strips were continuously immersed in physiological Krebs solution buffered containing (mM): 124 NaCl; 5.4 KCl; 1.2 $MgSO_4$; 11 HEPES; 11.7 Glucose and 2 $CaCl_2$. The pH of the solution was adjusted by using sodium hydroxide (NaOH) to 7.40. The high potassium solution (120mM) was prepared by isosmotic replacement of 120mM NaCl with KCl. Zero sodium solution (Tris substitution) containing 110 Tris base; 5.6 KCl; 1 $MgSO_4$; 10 HEPES; 10 Glucose and 2 $CaCl_2$. The pH of the solution was adjusted using HCl (10M) to 7.4. Zero calcium Krebs solution was prepared by omitting $CaCl_2$ and contained 2mM EGTA. Hank's solution (GibcoBRL; pH 7.4) containing 136 NaCl; 5.4 KCl; 4.17 $NaHCO_3$; 6.7 Na_2HPO_4; 0.44 KH_2PO_4;

5.5 glucose and 0.04 $CaCl_2$ then was bubbled with O_2. KB medium solution used during cell isolation containing 40 KCL, 10 K_2HPO_4, 10 taurine, 10 TES, 11 glucose, 5 pyruvate, 5 creatine, 0.04 EGTA, 100 K-glutamate, The pH of the solution was adjusted by using potassium hydroxide (KOH) to 7.40. Details on other stock solutions are given in the individual chapters.

2.3 Simultaneous measurements of force and intracellular calcium

2.3.1 The fluorescent indicator of free cytosolic calcium - Indo-1

Indo-1 is a ratiometric dye that can be used to indicate the change s in intracellular calcium concentration. The indicator is excited by UV light at a wavelength of 355nm and emits light at a specific wavelength, which depends upon whether or not it is bound to calcium. In the absence of calcium, Indo-1 emits more 500nm wavelength light, and when calcium is bound to the molecule, Indo-1 emits more 400nm wavelength light. The ratio of these two wavelengths gives an indication of the change in intracellular calcium in the cells. However, it is not an indicator for absolute cytosolic calcium concentration. Since a ratio of the two wavelengths is used, any additional effects of the concentration of the indicator, the illumination intensity and any movement artifacts can be ignored (Cobbold & Rink, 1987). In this study, Calcium sensitive dye Indo-1 acetoxymethyl ester (Indo-1 AM, Molecular Probes, Oregon, USA) was used to measure the intracellular free cytosolic calcium. Indo-1 AM is a cell membrane permeable form of indo-1 so that it can help indo-1 indicator diffuse passively across cell membranes. After it enters into the cells, the AM esters can be cleaved from indo-1 AM by cytosolic esterases.

2.3.2 Tissue loading with Indo-1 AM

Indo-1 AM was supplied in the anhydrous form (50μg vials) and made into a 1mM stock solution dissolved in 50μl of dimethyl sulphoxide (DMSO) containing 20% (w/v) of non-ionic detergent pluronic acid (F-127 Molecular Probes, Oregon, USA) to mix the indo-1 AM in aqueous solutions. A 20μM indo-1 AM solution was used to load the ureter by adding 40μL of the indo-1 AM stock solution containing the pluronic acid to 2ml of Krebs solution. The dissected ureter strips were then loaded on a rotator for 3 hours at room temperature. After loading, the ureters were washed with Krebs solution to remove any excess Indo-1 AM prior to experiments. Then the ureteric strip was cut into 3-4 mm strips and clipped with aluminum foil shaped clips.

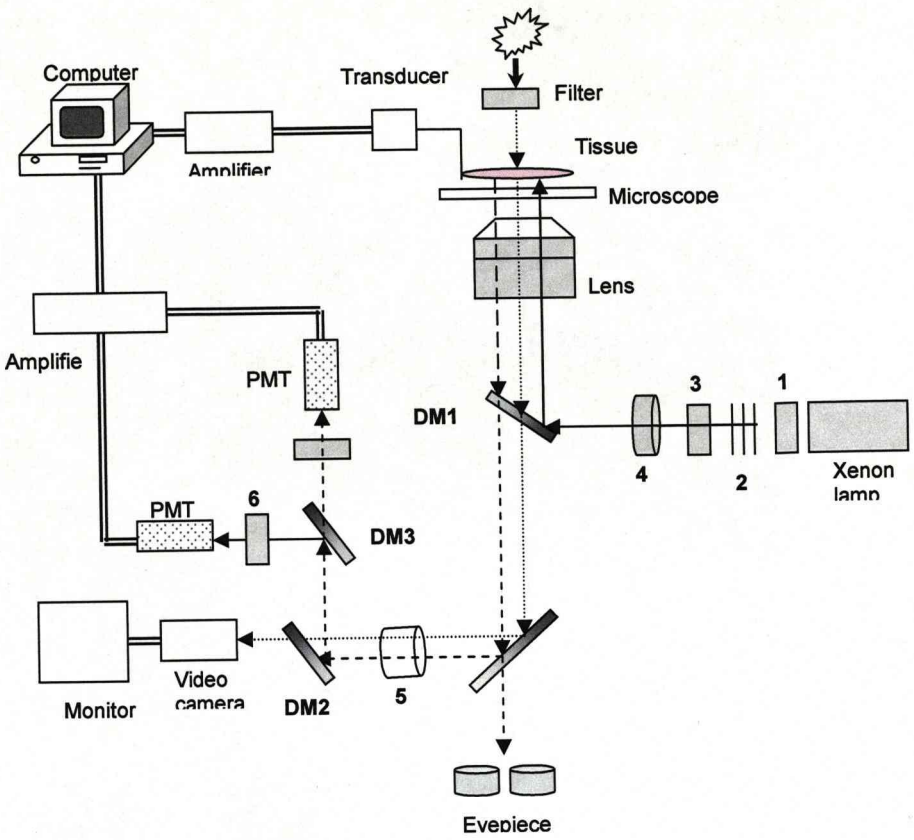


Figure 2.1 Schematic diagram of apparatus designed for simultaneous measurement of force and calcium. Ultraviolet illumination is provided by xenon lamp while the heat filter (1) and neutral density filter (2) block excess heat and reduce the light intensity, respectively. The appropriate wavelength of light at 355nm was selected by the interference filter (3) the mechanical shutter (4) is used to ensure the tissue is only illuminated for the minimum amount of time required. The light is reflected upwards towards the tissue by a dichroic mirror (DM1) and focussed onto the preparation by a lens. The emitted light then passes back through the lens and dichroic mirror and is reflected through a sliding mirror through a diaphragm (5) onto another dichroic mirror (DM2). Light with a wavelength longer than 610 nm is directed to a video camera that can be used to produce an image of the tissue on monitor, light with a wavelength less than 610 nm is directed to a third dichroic mirror (DM3). The light is spilt by a DM3, passing to either the 400 nm or 500nm photomultiplier tubes (PMT). In front of each PMT there is an emission filter at the appropriate wavelength (6). Figure designed by Dr S. Shabir.

2.3.3 Simultaneous measurement of force and calcium

The experiment was performed in a Faraday cage in a dark room to reduce the electrical interference and to avoid background illumination. The ureter strips loaded with Indo-1 were placed in the apparatus shown in Figure 2.1 in order to perform the experiment to simultaneously measure the force and intracellular calcium. One end of the ureteric strip with clips was attached to a fixed hook and the other end was placed over a force transducer (Grass FT03, Massachusetts, USA) in a constant perfusion organ bath. The ureteric strip loaded with Indo-1 can be excited at 340 nm and emitted light at wavelength of 400nm and 500nm was collected. The ratio of these emissions was used to estimate changes in intracellular calcium.

The ureteric strips loaded with indo-1 were mounted in a small chamber over the stage of an inverted microscope (Nikon Diaphot, USA) equipped with a 20 x Fluor objective. Experiment on the guinea pig ureteric smooth muscles was routinely performed at 33°C; all the experiment on rat ureter was performed at room temperature. The bottom of the chamber was sealed with a glass coverslip using high vacuum grease (Dow Corning). The tissue was continuously perfused with buffered Krebs solution (pH 7.4) at a flow rate of $4ml.min^{-1}$. When performing an experiment in which a solution with a high potassium concentraction was used to stimulate the muscle, a flow rate of 9 $ml.min^{-1}$ was used to ensure that the flow of solution in the organ bath occurred as quickly as possible. Phasic contractions were induced by electrical field stimulation (EFS) using Ag/AgCl electrodes with rectangular pulses of amplitude 5-7V and 100msec

duration at around 40 seconds intervals. The optimal length (L_0) of the muscle strip was determined by stretching the muscle to a passive force which was 20-30% of the force generated during high K^+ response. Normally at this length, the preparations showed maximum force responses.

UV light emitted by a xenon lamp, filtered at wavelength of 355nm, was used to excite the Indo-1 Ca^{2+} sensitive indicator in the tissue. The light emitted by the Indo-1 loaded tissue was obtained at wavelengths 400nm and 500nm and monitored via photomultiplier tubes and changed into ratio by using a Digidata 1200 board and Axoscope 8 software (Axon instruments) on the computer. The increase in intracellular calcium concentration shift the indo-1 fluorescence spectrum to UV direction and produce an increase in fluorescence at the wavelength of 400 nm and a decrease at the wavelength of 500 nm. Only when the two wavelength move in the opposite direction the signal was used. The force and calcium signal were recorded at a sampling rate of 100Hz. This was to ensure that rapid changes in intracellular calcium and force could be recorded correctly. Figure 2.2 shows a typical trace of the record to simultaneously measure force and intracellular calcium. The ratio of the emitted light at 400nm and 500nm wavelength (F_{400}/F_{500}) is shown, which indicates the change in intracellular calcium.

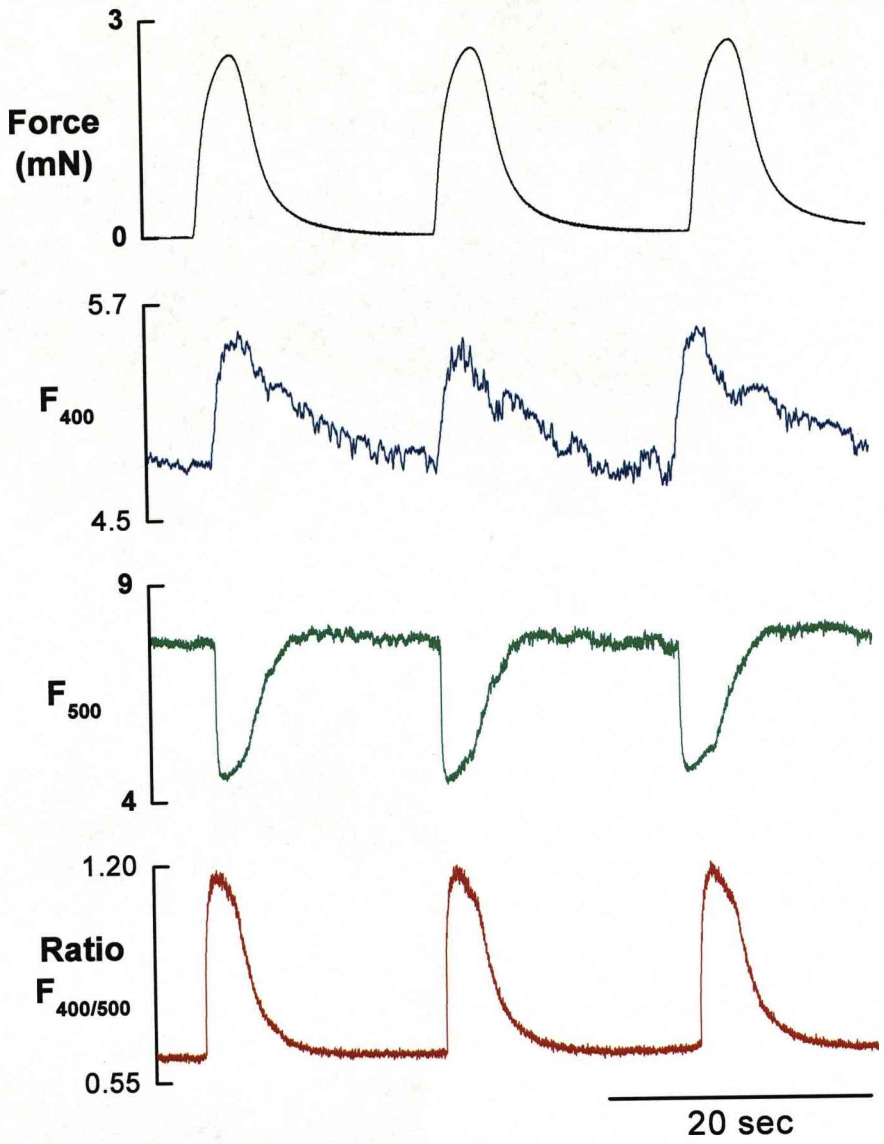


Figure 2.2 Simultaneous measurement of force and intracellular calcium. Force (black) and fluorescence emissions of ureter tissue loaded with Indo-1 wavelengths of collected light at 400nM (green) and 500nM (blue) were recorded. Change in intracellular calcium (red) obtained as the ratio of 400/500 emissions.

2.3.4 Calibrating the force

Force obtained from the experiment was measured in Newton (N). The force transducer converted the force of contraction of the smooth muscle into an electrical signal that was recorded on a computer using Axoscope software. The force was calibrated by comparing the signal obtained from the transducer to force produced by a known weight. The signal obtained converted to force was done by using the equation $N=kg.m.s^{-2}$ where 1kg is 9.8N.

2.4 Identification and distribution of PKC isoform

2.4.1 Protein extraction and quantification

Rat and guinea pig ureters and samples from skeletal muscle, airway smooth muscle and brain tissue were dissected and rinsed and homogenized in protease inhibitor solution containing RIPA buffer (1 X PBS, 0.5% sodium deoxycholate, 0.1% SDS) at a volume of 3ml/g tissue, 0.1mg/ml PMSF, 1μM sodium orthovanadate and 30μl Aprotinin under ice. The samples were centrifuged at 10,000g for 10 minutes at 4°C then the supernatant was removed and recentrifuged at 10,000g for 10 minutes at 4°C and the supernatant was removed. The protein quantification was carried out using the BioRad protein Assay. The tissues were diluted on a range from 1:10 to 1:100 in RIPA buffer. The samples and standards (BSA+dH_2O) were mixed with the BioRad Dc working reagent with 20 μl reagent S per 1ml reagent A. Then the reagent B was added to the samples and standards and incubated at room temperature for 10 minutes. The protein concentration was calculated by comparing the 750nm light absorption through the unknown sample with the equivalent value on the graph of 750nm

absorption/BSA concentration using Microsoft Excel software.

2.4.2 SDS-PAGE

Protein extracts were mixed with RIPA buffer and solubilized in equal volumes of reducing sample buffer containing 0.5M Tris-HCl pH 6.8 (Sigma), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and heated at 95°C for 3 minutes. SeeBluePlus2 (Invitrogen) was used as molecular marker.

20µl of sample containing 50 µg protein was loaded per lane and was resolved using a 4% polyacrylamide stacking gel. The proteins were then separated on a 12% polyacrylamide gel. The gel was electrophoresed on a Bio-Rad Protean II system for 30 minutes first at a 80V until the sample ran through the stacking gel and then ran at 120V for about 1 hour until the blue dye reach the bottom of the gel. The running buffer containing 5mM Tris-Base(Sigma), 36.4mM Glycine(Sigma), 0.02%SDS(Sigma) and pH8.3 in a solution of 600ml. The PVDF membrane was pretreated in methanol for 10 seconds, washed in dH_2O and then left to equilibrate in transfer buffer for 10 minutes containing 250mM Tris-Base, 192mM glycine, 20% methanol. The protein was then transferred to PVDF at 55V for 1 hour. Then the PVDF was incubated in block solution containing 5% non-fat milk power, 0.1%TWEEN 20 in TBS overnight at 4°C.

The next day the PVDF was washed three times for 5 minutes in TBS with 0.1% Tween 20. The membrane was incubated with primary antibody PKC isoform α, β, δ and ε (BD Biosciences) diluted in blocking solution each being adjusted at the

optimized concentration for PKCα, β, δ and ε for 1 hour at room temperature. After washing 5 times for 5 minutes with PBS-0.1%Tween, the membrane was incubated with secondary antibody (rabbit anti-rat 1:5000) in blocking solution for 1 hour at room temperature. The membrane was then washed three times for 5 minutes in TBS Tween 20 and once in TBS for 5 minutes. The membrane was incubated in Supersignal West Pico Chemiluminescent Substrate and exposed to Hyperfilm ECL film (Amersham Biosciences) for 10 sec to 5 minutes and developed using a Kodak auto-developer (Kodak, Hemel Hempstead, UK). Positive controls airway smooth muscle and brain tissue and negative controls were also used.

2.4.3 Immunohistochemistry

Tissue was placed in neutral buffered formalin for 24 hours. Then the ureter was placed in PBS to prevent over-fixation of formalin. Ureter was then placed in processor, passed through different levels of formalin and alcohol and then was embedded in was. Thin sections were obtained by using microtone.

The slides were incubated in xylene for 30 minutes at room temperature to remove the paraffin wax from the tissue, followed by rehydration in a series of freshly made alcohol baths at 100%, 95%, 85%, 70% and 50% and washed in distilled water for at least 5 minutes. Antigen retrival was performed by placing slides into the heated 0.01M citrate buffer at pH 6 and boiling for 12 minutes. The slides were then transferred to distilled water for 5minutes and incubated with the dual endogenous enzyme block from the DAKO envision kit (DAKO Cytomation)

for 20 minutes. After this, slides were rinsed in distilled water and washed for 15 minutes in wash solution bath (TBS with 0.05% Tween 20). The slides were incubated with wash solution containing BSA albumin for 30 minutes. Then the primary antibody PKC α, β, δ, ε (1:100 dilution each) (BD Biosciences) was diluted in TBS-Tween with 1% bovine serum albumin solution, added onto the slides and incubated overnight at 4°C. The next day, the slides were rinsed TBS-Tween solution three times for 5 minutes with gentle agitation, and then incubate with DAKO Envision polymer (DAKO Cytomation) and then exposed to DAB substrate (DAKO Cytomation) for 10 minutes. The image was captured using Nikon MicrophotFX microscope fitted with a Nikon DXM1200 digital camera.

2.5 Confocal imaging of isolated ureteric myocytes

2.5.1 Cell isolation

After cleaning the ureters were cut into small pieces and placed into Ca^{2+} free Hanks solution and used for cell isolation. The pieces of ureter for cell isolation were washed with Hanks solution at 35°C twice for 15 minutes. After washing, the ureteric strips were transferred into solution containing collagenase Type I (0.3 mg/ml), protease (0.15 mg/ml), trypsin inhibitor (0.2 mg/ml) and BSA (1 mg/ml), for 40 minutes at 35°C. The tissue was then placed in KB media and after 15 minutes was triturated by a fire-polished Pasteur pipette. Dispersed cells were filtered, centrifuged at 2000 rpm for 2 min and then placed into fresh KB media containing 5μM Fluo-4 AM and Pluronic F-127 for loading for 30 minutes at room temperature. After loading cells were centrifuged and placed into dye – free

KB media and kept for 30 minutes at room temperature and then refrigerated until use. Cells were excited at 488 nm using Argon –Krypton gas laser and Fluo-4 fluorescence was measured at 510nm and used as a measure of $[Ca^{2+}]_i$, Changes in fluorescence were expressed as a pseudo-ratio of F/F_0 where F_0 is the background fluorescence and F is the changes of fluorescence in the region of interest where cytoplasmic Ca^{2+} was increased.

2.5.2 Confocal imaging

The confocal images were taken on a Perkin Elmer, Nipkow disc-based UltraView confocal system linked to a fast digital camera and computer workstation running Ultra View LCI software attached to an Olympus inverted microscope. Images were aquired using x60 water-immersion objective lense (NA 1.2 at 20–50 frames s^{-1}).

2.6 Determination of myosin light chain phosphorylation

2.6.1 Freezing apparatus

The freezing apparatus was designed by Dr T. Burdyga. As shown in Figure 2.3, it consists of a chamber containing six wells so that each well could be filled with different solutions. The chamber is fabricated from a block of Perspex that has been drilled to produce six large holes in a horizontal arrangement. Wells made of plastic were placed into each hole and glued into place to create a watertight seal. A series of tubes connected four of the holes so that with the aid of a pump, hot water could be circulated around the base of four wells of the wells to raise the

temperature of the solution within the wells to the required experimental temperature ("a" in Figure 2.3A). The base of the chamber had four prongs in each corner over which springs were placed ("a" in Figure 2.3). The prongs slotted into four corresponding hollow cylinders mounted on the bottom of the chamber ("b" in Figure 2.3). This system allowed the freezing chamber to be lowered, moved along, and lifted back up by the user. On one side of the chamber was an open round shaped holder into which the glass container holding the pre-cooled acetone could be placed ("c" in Figure 2.3). This was made of metal to prevent any damage by acetone that may be spilt upon it.

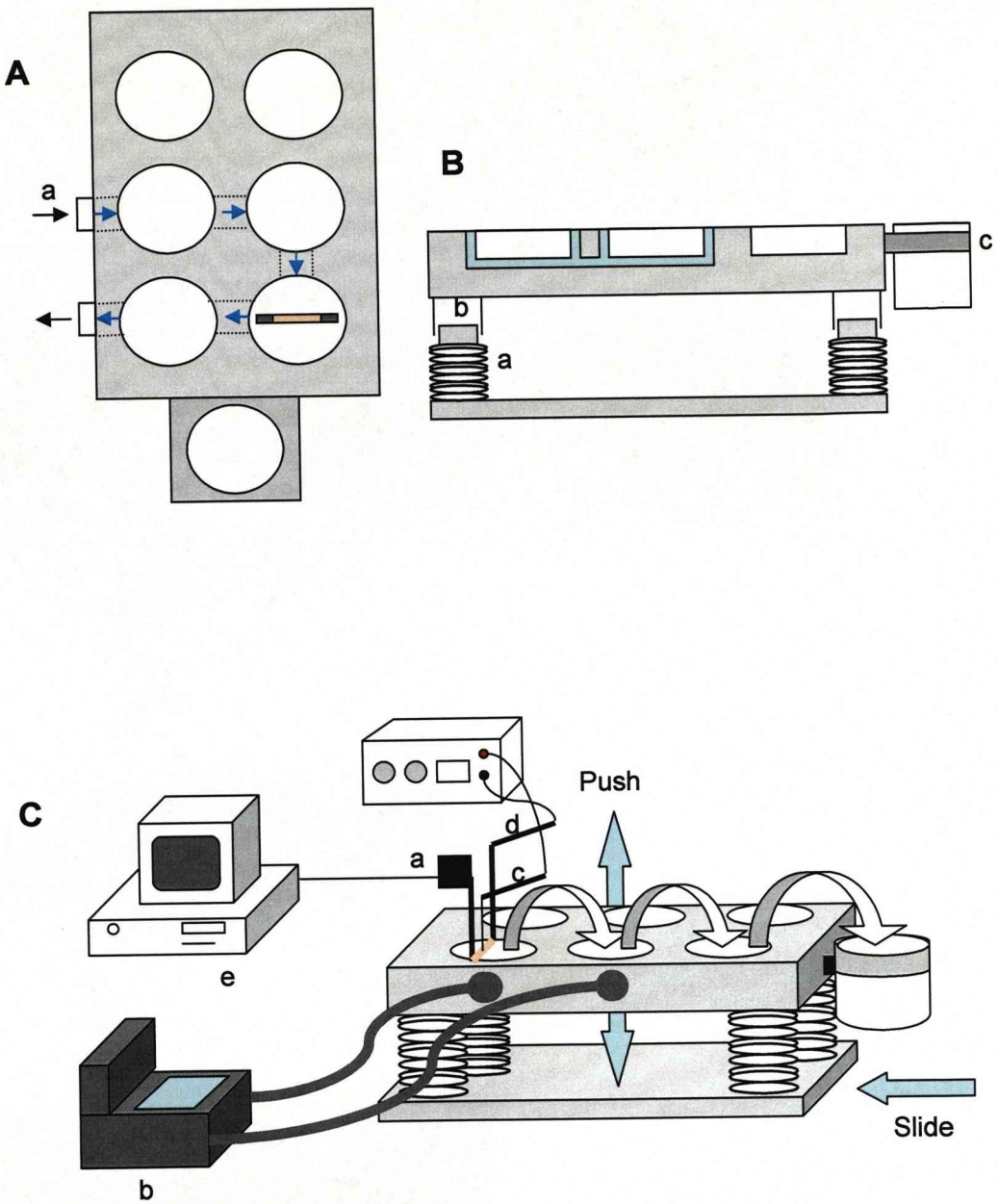


Figure 2.3 Diagram of apparatus designed to freeze the ureter. The freezing chamber showing the connections of tubes, electrodes and force transducer.

Figure designed by Dr S. Shabir.

2.6.2 Freezing the ureter

Two ureters obtained from one animal were each cut into 3-4mm strips, giving approximately 16 pieces of ureter from each animal. The two ends of each strip of ureter were fitted with aluminium foil clips. One clip was attached to a fixed hook, and the other was attached to a force transducer (Grass FT03 force transducer, "a" in Figure 2.3). The strip of ureter was put in about 400μl bath solution in one well of the freezing chamber. The tissue can be sequentially immersed in different solutions moving from well to well by pushing down, moving along and releasing the chamber back up so that the tissue can be immersed in another solution contained in the next well. This method allows for a quick change of the solution bathing the ureter preparation.

The preparation can be frozen at any desired point of the force. The acetone was pre-cooled at -78°C, and then the tissue at any point of the force development was quickly immersed into the vessel of acetone and snap frozen. The time taken to transfer the preparation from the bath solution to acetone is about 200msec. The time taken for all of the phosphorylation in the muscle to be arrested ≈200msec (Maass-Moreno *et al.*, 2001). The frozen point is recorded on the computer which is shown in Figure 2.3. Figure 2.4 shows a representative trace of force produced by a freezing preparation. Firstly, the ureter was immersed in a well containing Krebs solution and transferred to a well containing High K^+ solution for 40 seconds. It was then immersed in zero calcium solution for 80 seconds before being put into carbachol solution for 30 seconds and returned to the zero calcium solution. The protocol was also repeated after incubation in Krebs solution

containing 5μM Ro320432 for 10 minutes. While in carbachol solution, the tissue generated force. At different points along the force development process, the tissue was quickly frozen in the pre-cooled acetone. Figure 2.4. shows representative trace during freezing. After the tissue was quickly frozen, the samples were transferred to eppendorfs containing a solution of acetone mixed with 5% (w/v) trichloroacetic acid and 20mM dithiothreitol (DTT) kept at -80°C by cooling in dry ice. All the samples were stored at -75°C in the freezer for at least 24 hours.

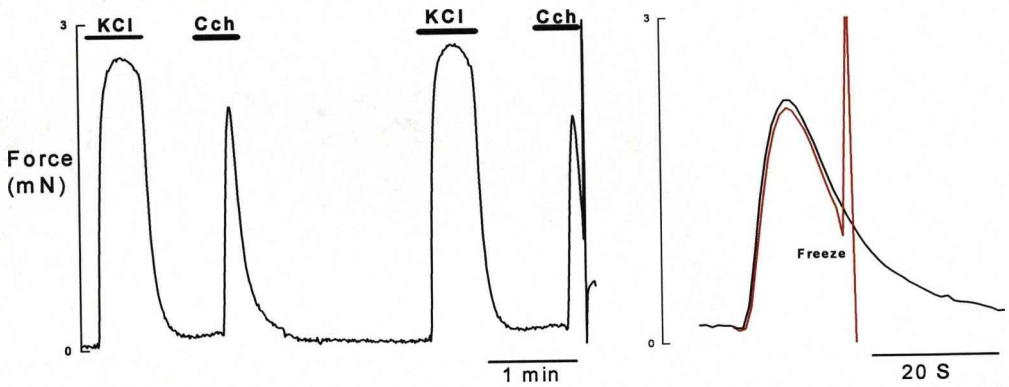


Figure 2.4 Typical trace during a freezing experiment. The force recorded during an experiment in which carbachol was used to stimulate the ureter. The left panel shows a control contraction when the tissue was frozen. The right panel shows the force produced when the tissue was frozen superimposed over the control force.

2.6.3 Protein extraction

Samples were thawed at room temperature before use and the acetone solution was discarded. Then the samples were washed twice with 1ml of an acetone and 20mM dithiothreitol solution to remove the trichloroacetic acid. The samples were washed by rotating for 30 minutes. Each strip of ureter was taken out of the eppendorf and the aluminium foil clips were removed from the ureter. Then each strip of ureter was placed in eppendorf tubes containing 100µl of 9 M urea extraction buffer containing (mM): 20 DDT; 10 EGTA; 1 disodium EDTA; 5 NaF; 10 phenylmethylsulfonyl fluoride; 2 Tris base; 2.1 glycine and 0.04% bromophenol blue. The eppendorfs were placed on a blood tube rotator and inverted for 1.5 hours. Every 15 minutes each eppendorf was vortexed for 15 seconds. Then, the samples were centrifuged at 10,000g for 30 minutes. The samples were then used immediately or stored in the freezer (-20°C).

2.6.4 Electrophoresis

20µl of sample was loaded per lane and was resolved using a 3% acrylamide-urea stacking gel. The proteins were then separated on a 10% polyacrylamide-glycerol gel. The gel was run using Bio-Rad Protean II system for 30 minutes first at 10mA until the sample running through the stacking gel and then running at 16mA until 30 minutes that the blue dye is about to exit the separating gel. The running buffer containing 100mM glycine and 50mM Tris base and had a pH of 8.7. 250µl of 2-mercaptoethanol was added to the upper tank buffer after the samples were loaded.

The protein was then transferred to a nitrocellulose paper running at 300mA for 1 hour by using semi-dry transfer technique. The filter paper and nitrocellulose paper were soaked in semi-dry transfer buffer containing 25mM Tris-HCl, 192mM glycine and 20% methanol at pH 8.3. After the protein transfer, the membranes were blocked in Tris-buffered saline containing 0.1% polyoxyethylenesorbitan monolaurate (Tween 20) at pH 7.6 (TBS-T) and 3% non-fat dry milk overnight at 4°C.

The next day the membrane was washed in TBS with 0.1% TWEEN 20 three times and each time for 5 minutes. The membrane was then incubated with monoclonal mouse anti-myosin light chain antibody (1:250) for 2 hours in TBS-T containing1% non-fat dry milk at room temperature. After washing with 5 times TBS-T 1% milk for 10 minutes each at room temperature, the membrane was then incubated with secondary biotin-conjugated anti-mouse antibody (1:1000) in TBS-T 1% milk for 40 minutes at room temperature, and then washed 3 times in TBS-T each time for 5 minutes. Then the membrane was incubated for 40 minutes with streptavidin-horseradish peroxidase conjugate (Amersham; 1:5000) and then washed in TBS-T twice for 10 minutes followed by one wash in TBS for 10minutes.

The membranes were incubated with Supersignal™ CL-HRP Substrate System (Pierce Chemical Co) for 5 minutes as described by the manufacturers, after which lumigrams of the western blots for non-phosphorylated and phosphorylated MLC_{20} were obtained by exposure to Amersham Hyperfilm™ECL™ and developing using Kodak developer and fixing solutions.

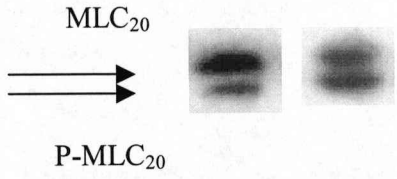


Figure 2.5 Typical lumigrams of western blots. The non phosphorylated MLC_{20} band is at the top and the mono-phosphorylated band P-MLC_{20} is shown at the bottom. The percentage of MLC_{20} phosphorylation level can be calculated by the ratio of the density of the lower band to the total density of both the lower and top bands.

2.6.5 Measurement and quantification of protein expression

Figure 2.5 shows a representative western blot obtained. The upper band shows the unphosphorylated myosin light chains and the bottom band shows phosphorylated myosin light chains. The percentage of myosin light chain phosphorylation level can be determined by the ratio of the density of the phosphorylated band to the sum of the densities of both phosphorylated and unphosphorylated bands, multiplied by 100 as following equation:

$$\left(\frac{\text{density of phosphorylated band}}{(\text{density of phosphorylated band} + \text{density of unphosphorylated band})}\right)\times 100$$

The density of each blots can be measured by scanning the blots with a Hewlett Packard Scanjet 4C and the density of the bands measured using the Scion software.

2.6.6 Analysis and statistics

The statistical significance of the results was measured using the appropriate t test.

P values <0.05 were taken as statistically significant. One-Way ANOVA test was applied to test the significant difference ($P<0.05$) between different groups. Most of the results are given as a percentage of the control value unless otherwise stated in the individual chapters.

Chapter 3

Identification and investigation of functional role of PKC in ureter smooth muscle

Chapter 3

Identification and investigation of functional role of PKC in ureter smooth muscle

3.1 Introduction

PKC consists of a family of serine/threonine kinases with at least 12 members, grouped into three types-conventional, novel and atypical PKCs. Its distribution and function varies significantly in cells and tissues of different species. Overall PKC isoforms have different functional roles such as regulation of cell growth, apoptosis, ion channel activities and smooth muscle contractions (Rasmussen *et al.*, 1987; Throckmorton *et al.*, 1998; Gutcher *et al.*, 2003; Barman *et al.*, 2003). If PKC isoforms are to be considered as therapeutic targets for smooth muscle contraction disorder, it is important to know which isoforms are expressed by particular type of the smooth muscle. PKC α and ε have been shown to express in guinea pig airway smooth muscle (Fatma, *et al.*, 2001). The presence of PKC α, β and ε has been demonstrated in rat skeletal muscle (Yamada, *et al.*, 1995). In addition, PKC δ has been found in rat brain and expressed exclusively in guinea pig gastrointestinal tract smooth muscle contributing to the control of tonic contraction (Garciam *et al.*, 1993; Poole *et al.*, 2003, 2006). PKC is involved in the regulation of contractile activity in a variety of smooth muscles (see Chapter 1). Activation of PKC causes hyperactive contractility of vascular smooth muscle, contributing to the development of

cardiovascular diseases (Salamanca & Khalil, 2005). Increased PKC activity in diabetic human penile tissue has also been reported (Angulo *et al.*, 2006).

Phorbol ester (PDBu) is a widely used PKC activator. PKC activators have potent contractile effects on aortic smooth muscle (Jin *et al.*, 2008). Activation of PKC increased excitability and altered patterns of gut motility (Horowitz *et al.*, 1999). Certain phorbol ester act via PKC to decrease Ca^{2+} current via Ca^{2+} channel (Doemer *et al.*, 1990). PKC specific inhibitor Ro320432 has been widely used to investigate functional role of PKC. Enhanced PKC activity contributes to increased vascular reactivity in disease states. Therefore, PKC inhibitors may be potentially used as therapeutic agents to treat some of these disorders.

Rottlerin, also referred as mallotoxin, has been used as a PKC δ specific inhibitor in some studies(Barman *et al.*, 2006; Sakai *et al.*, 2008) and has been used to identify the functional role of PKC δ in control of contraction of pulmonary artery (Barman *et al.*, 2004) and vascular smooth muscle cell migration (Kamiya *et al.*, 2007). Studies also showed that rottlerin directly activated several types of K^+ channels including BK_{Ca} channel (Zakharov *et al.*, 2005).

In this study, we used immunohistochemistry and western blotting to identify the presence and distribution of several PKC isoforms in the guinea pig and rat ureter. In addition, we used PKC activator PDBu and inhibitor Ro320432 to investigate

functional role of PKC in control of excitation-contraction coupling in ureter smooth muscle of both species by using different experimental protocols and modes of stimulation.

3.2 Materials and methods

Simultaneous measurement of calcium and force

The simultaneous measurement of force and intracellular calcium were described in detail in Chapter 2.

Krebs solution was prepared as described in Chapter 2. The tissue was continuously perfused with Krebs, Krebs containing PDBu (0.1μM) and Ro320432 (5μM).

The ureter smooth muscle was stimulated electrically at interval of about 40 seconds to produce phasic contractions associated with Ca^{2+} transients by using Ag/AgCl electrodes placed in the bath solution with rectangular pulses (3-5V, 200ms).

Western blotting

The method of western blotting was described in detail in Chapter 2. Proteins were extracted from guinea pig and rat ureter muscle tissue in protease inhibitor solution containing RIPA buffer solution and after centrifuging at 10,000g for 10 minutes twice the supernatant was run on 4% polyacrylamide stacking gel then separated on a 12% polyacrylamide gel. Then proteins were transferred to a PVDF membrane and were blocked in solution containing 5% non-fat milk powder overnight. Next day, the membrane was incubated with antibodies against PKC α, β, δ and ε for 1 hour at room temperature. After washing out the first antibodies, the membrane was incubated with

secondary antibody for 1 hour. After washing out the secondary antibody, proteins were exposed to Hyperfilm ECL film (Amersham Biosciences) for 10 seconds to 5 minutes and developed using a Kodak auto-developer (Kodak, Hemel Hempstead, UK).

Immunohistochemistry

The method of immunohistochemistry was described in detail in Chapter 2. Generally, the slides were incubated in xylene for 30 minutes at room temperature then followed by rehydration in a series of freshly made alcohol baths 100%, 95%, 85%, 70% and 50%. Then the slides were placed in heated 0.01M citrate buffer and continued boiling for 12 minutes and was incubated with the dual endogenous enzyme block from DAKO kit for 20 minutes and washed in wash solution. The slides were then incubated with wash solution containing BSA albumin for 30 minutes. And then incubated with the primary antibody against PKCα, β, δ and ε overnight at 4°C. The slides were rinsed and then incubated with DAKO Envision Polymer and then exposed to DAB substrate for 10 minutes. Image was captured using Nikon MicrophotFX microscope fitted with a Nikon DXM1200 digital camera.

Chemicals

All chemicals were purchased from Sigma (Dorset, UK) except that PDBu and Ro320432 were purchased from Calbiochem.

The stock solutions were prepared as following method: PDBu was dissolved in DMSO at a concentration of 1mM. Ro320432 was dissolved in DMSO at a concentration of 5mM. The stock solution was diluted to the desired concentration with Krebs solution before starting the experiment.

Statistics

Data was analyzed with t-test, differences between means were assumed to be significant at $P<0.05$. One-Way ANOVA test was applied to test the significant difference ($P<0.05$) between different groups. All values represent mean ± s.e.m; *n* is the number of samples, each one from a different animal.

3.3 Results

3.3.1 Expression and distribution of PKC isoforms in the guinea pig and rat ureter

This work is the first to identify expression and distribution of four PKC isofoms α, β, δ and ε in the guinea pig and rat ureter by using western blotting and immunohistochemistry. PKC α and β are conventional Ca^{2+}-dependent PKC isoforms while PKC δ and ε are novel Ca^{2+}-independent PKC isoforms. PKC isoforms α, β, δ and ε have been identified by the bands with the molecular weight of about 84, 80, 78 and 90 kD, respectively. Western blotting shows that conventional PKC α and β are present in both guinea pig and rat ureteric extracts. PKC δ is found in the guinea pig but not rat ureter (Figure 3.1 and 3.2). PKC ε has not been found in ureter of both species. Positive control from skeletal smooth muscle, airway smooth muscle and brain from both animals have been used to check validity of our findings. Negative control has also been used to show the evidence of non-specific binding. The expression pattern is shown in Figure 3.1 and 3.2.

3.3.2 Distribution of PKC isoforms in guinea pig and rat ureter

Immunohistochemistry analysis of the PKC isofom was performed on guinea pig and rat ureteric tissue samples. The localization of PKC α, β, δ and ε was studied on fixed ureteric sections of both guinea pig and rat ureter.

Conventional PKC α was found in both smooth muscle and urothelium. Figure 3.1 and 3.2 show that it was more abundant in the urothelium of both species. Conventional PKC β was localized in smooth muscle and urothelium of the guinea pig ureter while in rat ureter it was confined exclusively to urothelium. Novel PKC δ was abundant in the guinea pig ureteric smooth muscle but not urothelium, while in the rat ureter it was not found at all (Figure 3.2). Novel PKC ε was completely absent in the ureter of both species. Negative control does not show any evidence of non-specific binding due to the secondary antibody and its enzyme conjugate. The summary of PKC isoform expression and distribution in the guinea pig and rat ureter is shown in Table 3.1. The immunohistochemistry data of both guinea pig and rat ureter are consistent with the western blotting results. However, collectively data obtained indicate that although PKC β was detected by western blotting analysis in both guinea pig and rat ureteric extracts, the immunohistochemistry analysis shows that this PKC isoform is only present in rat urothelium but not in smooth muscle suggesting that it can not be directly involved in control of rat ureter smooth muscle contractility.

In the next set of experiment, the functional role of PKC was investigated by studying the effects of PKC activator PDBu and PKC inhibitor Ro320432 on phasic contraction and Ca^{2+} transients in the guinea pig and rat ureter evoked by EFS.

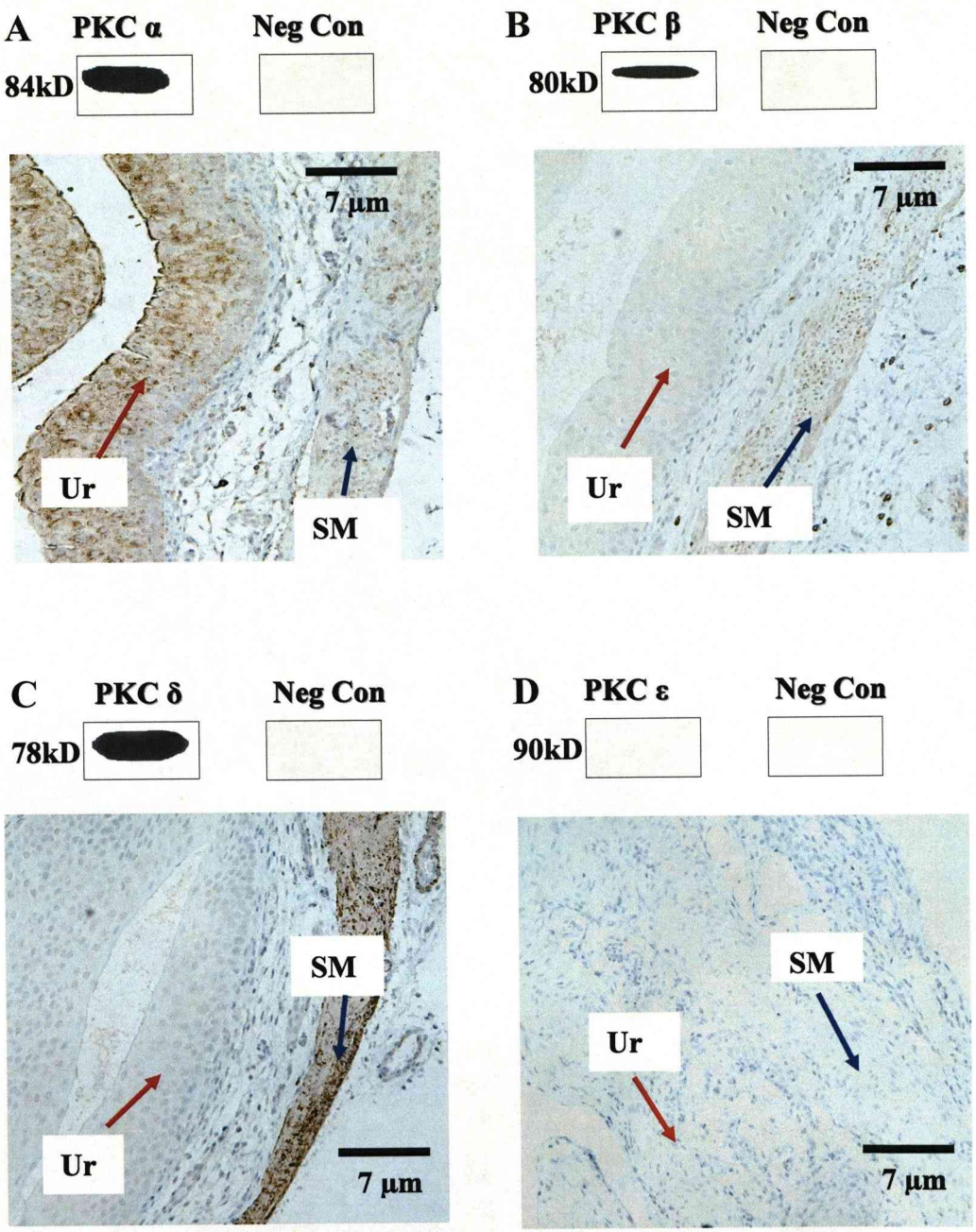


Figure 3.1 The expression and distribution of PKC isoforms in guinea pig ureter. (A-D) Western blots (top panel) and immunostaining of sections of ureter (bottom panel) showing expression and distribution of PKC isoforms α, β, δ and ε in the guinea pig ureter. Ur is urothelium, SM is smooth muscle cell.

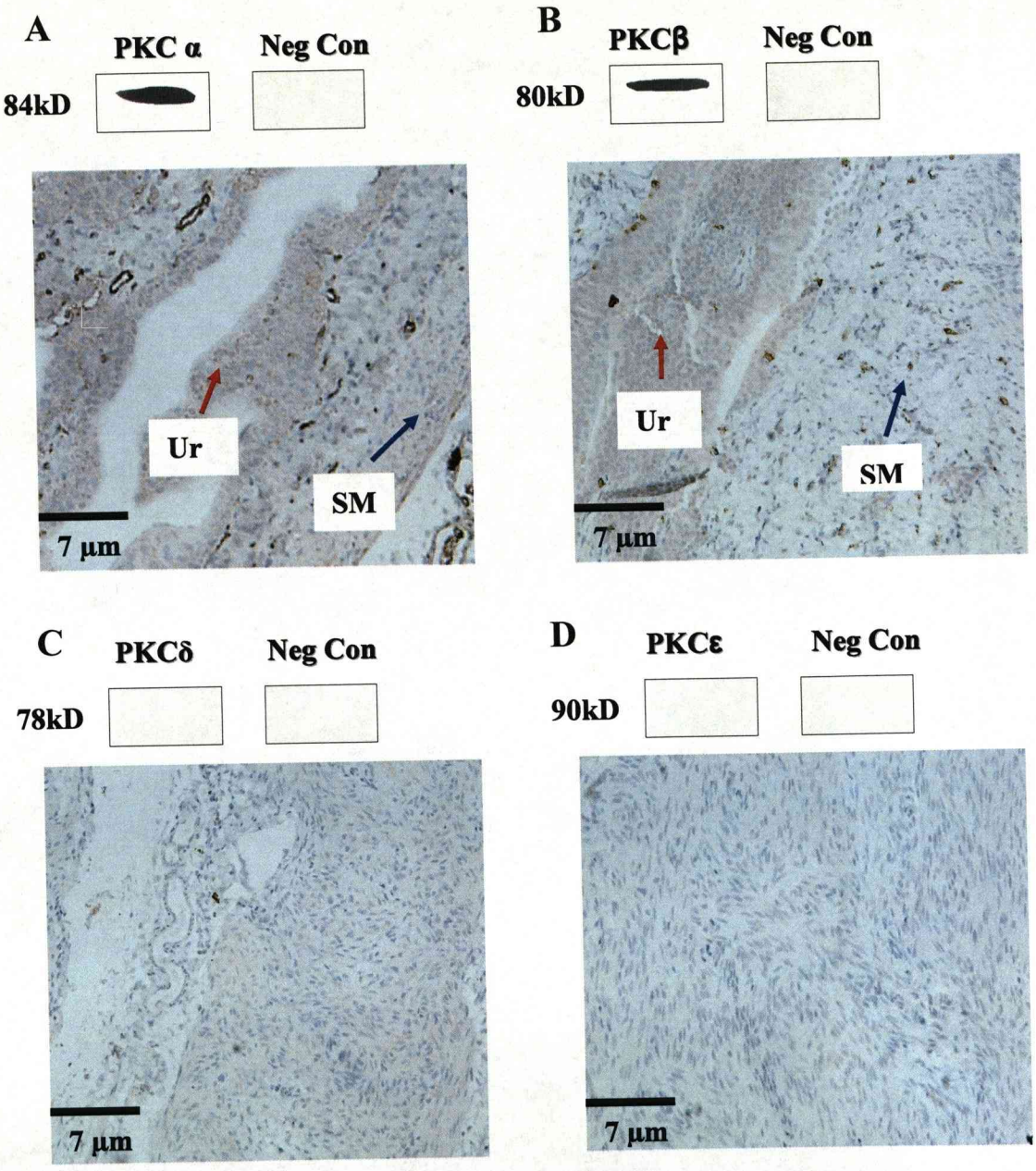


Figure 3.2 The expression and distribution of PKC isoforms in rat ureter. (A-D) Western blots (top panel) and immunostaining of sections of ureter (bottom panel) showing expression and distribution of PKC isoforms α, β, δ and ε in the rat ureter. Ur is urothelium, SM is smooth muscle cell.

Table 1. The distribution of PKC isoforms α, β, δ and ε in guinea pig and rat ureteric tissues

	PKC α	PKC β	PKC δ	PKC ε
G.P. ureter smooth muscle	+	+	+	
G.P. ureter urothelium	+	+		
Rat ureter smooth muscle	+			
Rat ureter urothelium	+	+		

3.3.3 The effects of PKC activator PDBu and PKC inhibitor Ro320432 on phasic contraction and Ca^{2+} transients in the guinea pig and rat ureteric smooth muscle evoked by EFS; Evidence for species dependence

In this set of experiments, the effect of direct activation and inhibition of PKC on phasic contraction and Ca^{2+} transients evoked by EFS have been studied in the guinea pig and rat ureteric smooth muscle loaded with Indo-1.

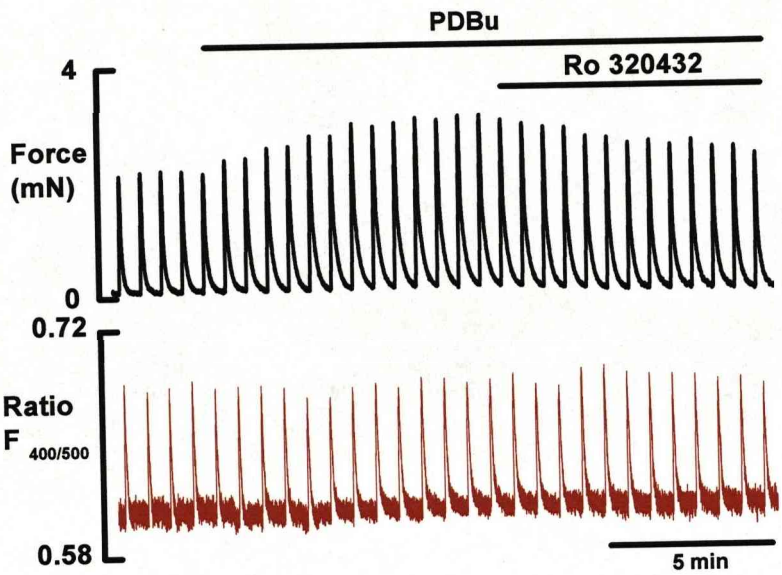
PDBu at concentration of 0.1μM was used in all experiments. PKC inhibitor Ro320432 was used in concentration of 5μM.

EFS of ureteric strips at about 40 second intervals produced a regular pattern of phasic contractions associated with Ca^{2+} transients in ureter smooth muscle of both species (Figure 3.3). Addition of PDBu produced stimulant effect on phasic contractions in both guinea pig and rat ureteric smooth muscle (Figure 3.3). In rat ureter a gradual rise in the baseline of force was also seen which despite the presence of PDBu was fully relaxed upon termination of stimulation (Figure 3.3 B). As can be seen from superimposed records of Ca^{2+} transients and force shown in Figure 3.4A, an increase in the amplitude of phasic contractions in the guinea pig ureter was associated mainly with an increase in the duration of the Ca^{2+} transients (1.3 ± 0.05 times, n=20) ($p<0.05$). In contrast, in the rat ureter an increase in the amplitude of force was not associated with any significant changes of the parameters of the Ca^{2+} transients

(Figure 3.4 B). The effects of PDBu on ureter smooth muscle were fully reversed by PKC inhibitor Ro320432 (Figure 3.3), which on its own had no effects on Ca^{2+} and force in ureteric muscle of both species (data not shown). Normalized traces of Ca^{2+} transients and force obtained in the absence and presence of PDBu clearly indicate that in the guinea pig ureter stimulant action of PDBu on force is caused by an increase in the duration of the Ca^{2+} transient (Figure 3.4 A) while in rat ureter it was Ca^{2+}-independent (Figure 3.4 B). The fact that in the rat ureter the relaxation phase of the phasic contraction was slowed down with no change in the kinetics of the relaxation of the Ca^{2+} transient suggests that the stimulant action of PDBu in rat ureter is Ca^{2+} independent and is likely to involve in Ca^{2+} sensitization mechanism. Ca^{2+}-independent elevation of baseline of force unaccompanied by changes in baseline $[Ca^{2+}]_i$ which fully relaxes on termination of stimulation (Figure 3.3 B) also suggests Ca^{2+}-independence mechanism of gradual build up of baseline force due to incomplete relaxation produced by each preceding phasic contraction in the rat ureteric smooth muscle. PKC inhibitor Ro320432 and PKC δ specific inhibitor rottlerin (data not shown) fully reversed stimulant action of PDBu in the ureter of both species (Figure 3.3). These data suggest that at least in ureteric smooth muscle rottlerin acts as a non-specific PKC δ inhibitor. This finding is consistent with some recent studies (Soltoff, 2007).

These data collectively indicate that guinea pig and rat ureter express different isoforms of PKC and respond differently to PKC stimulation by PDBu.

Guinea pig ureter



Rat ureter

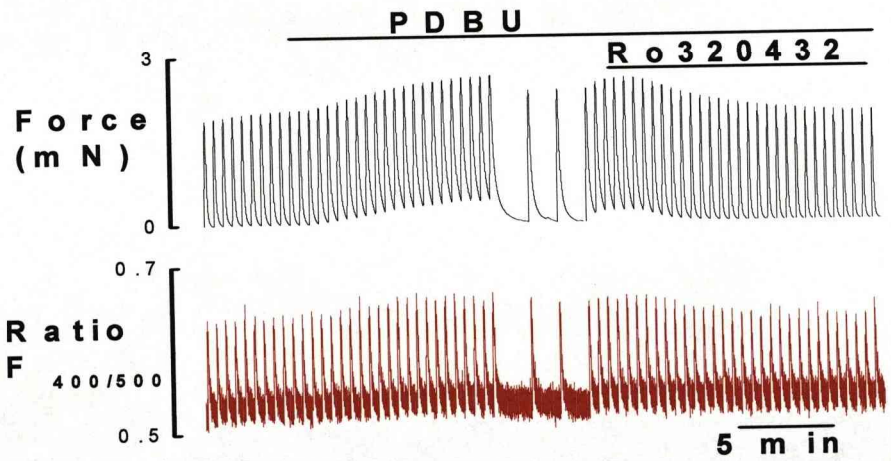


Figure 3.3 The effect of PKC activator PDBu and PKC inhibitor Ro320432 on Ca^{2+} transients and phasic contractions evoked by EFS in guinea pig and rat ureter smooth muscle. In this and all other figures bottom trace (red) shows change in $[Ca^{2+}]_i$ and top trace (black) represents force.

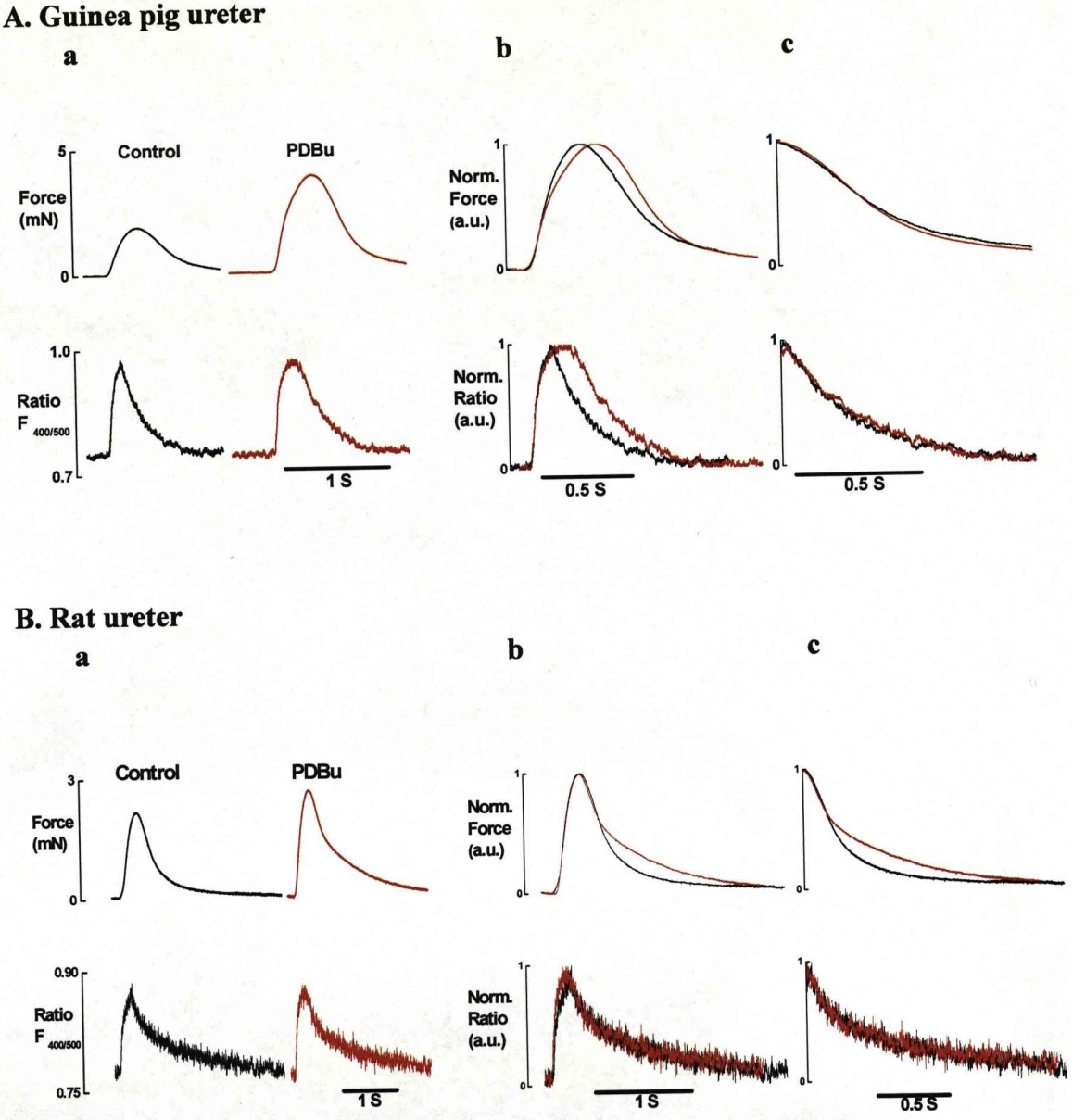


Figure 3.4 The effect of PKC activator PDBu on Ca^{2+} transients and phasic contractions evoked by EFS in guinea pig and rat ureter smooth muscle. Aa, Ba- independent Ca^{2+} transients and phasic contractions of guinea pig and rat ureter smooth muscle in the absence and presence of PDBu, respectively. Ab, Bb- superimposed records from Aa and Ba. Ac and Bc- superimposed relaxation phase of force and Ca^{2+} transient from Ab and Bb. In this figure, traces in red are records obtained in the presence of PDBu; traces in black are controls. Note the relaxation phase of rat ureter phasic contraction is slowed down by PDBu with no change of Ca^{2+}.

3.4 Discussion

The increase in $[Ca^{2+}]_i$ is required for activation of smooth muscle contraction. The relationship between $[Ca^{2+}]_i$ and force has been well established in ureter (Burdyga & Wray, 1999). There is a marked hysteresis between $[Ca^{2+}]_i$ and force, i.e. Ca^{2+} rises and peaks before force; there is no steady state between $[Ca^{2+}]_i$ and force during development of the phasic contraction. Thus, the duration of the Ca^{2+} transients plays a key role in control of the amplitude of phasic contraction (Burdyga & Wray, 1999). The longer the duration of Ca^{2+} transients, the higher is the amplitude of force. It suggests that equilibrium between Ca^{2+} transient and contractile element during the development of the phasic contraction is not reached.

PKC activator PDBu increased the amplitude and duration of phasic contraction evoked by EFS in both guinea pig and rat ureter smooth muscle. PKC specific inhibitor Ro320432 could fully reverse the stimulant effects of PDBu on ureters of both species. In the guinea pig ureter an increase in the amplitude and duration of phasic contraction was caused mainly by an increase in the duration of the Ca^{2+} transient while in rat ureter it was Ca^{2+}-independent.

In rat ureter, activation of PKC is involved in regulation of relaxation phase of phasic contraction by prolonging the relaxation time of the phasic contraction without any significant changes in the kinetics of intracellular $[Ca^{2+}]_i$. In other words, PKC is

modulating smooth muscle contraction in a Ca^{2+}-independent manner. This suggests that the major role of PKC in rat ureter smooth muscle contraction is modulation of Ca^{2+} sensitization by affecting the activity of MLCP. In the guinea pig ureter, Ro320432 at 5μM could reverse the stimulant effect of PKC and bring the parameter of force and Ca^{2+} to control level which proves it to be a potent and efficient PKC inhibitor in both guinea pig and rat ureter as reported by others in other types of smooth muscle (Mauro *et al.*, 2002; Kim *et al.*, 2004).

Thus, there are two species dependent mechanisms involved in modulation of force by PKC in ureter smooth muscle. Based on difference in PKC isoforms expression between the two species, one can suggest that in the guinea pig ureter smooth muscle PKC β and δ are more functionally important. One of these or both isoforms might be involved in modulation of the duration of the Ca^{2+} transient which underlies the increase in the amplitude of force. In contrast, rat ureter smooth muscle mainly express PKC α isoform which is likely to be involved in modulation of force in Ca^{2+}-independent manner by inhibiting MLCP activity as was reported for some other types of smooth muscles (Li *et al.*, 1998; Webb *et al.*, 2000; Hai *et al.*, 2002). Possible role of other PKC isoforms which were not investigated in this study may contribute to regulation of the force-Ca^{2+} relationship in ureteric smooth muscle can not be excluded.

In conclusion, we are the first to examine the expression and distribution of PKC

isoform in guinea pig and rat ureter by using western blotting and immunohistochemistry. We also found for the first time that PKC activator PDBu could increase the amplitude and duration of force and duration of Ca^{2+} transient in the guinea pig ureteric smooth muscle and an increase the amplitude and duration of force without changes of $[Ca^{2+}]_i$ in the smooth muscle of the rat ureter. PKC inhibitor Ro320432 reversed the stimulant effects of PDBu in the ureteric smooth muscle of both species. It shows Ca^{2+}-dependent mechanism in the guinea pig ureter and Ca^{2+}-independent mechanism in the rat ureter.

Chapter 4

Mechanism of the stimulant action of PKC activation in the guinea pig ureter smooth muscle

Chapter 4

Mechanism of the stimulant action of PKC activation in the guinea pig ureter smooth muscle

4.1 Introduction

According to literature, PKC can produce stimulant action via inhibiting Ca^{2+} sparks/STOCs coupling mechanism in vascular smooth muscle (Bonev *et al.*, 1997). Ca^{2+} sparks is spontaneous transient localized Ca^{2+} release from the SR via a cluster of ryanodine-sensitive Ca^{2+} release channels (RyR). Depending on the types of smooth muscle, Ca^{2+} sparks can activate BK_{Ca} channel on the plasma membrane to generate STOCs, Ca^{2+}-activated Cl^- channels to generate STICs or both populations of channels to generate STOICs (Nelson *et al.*, 1995; Bonev *et al.*, 1997; Jaggar *et al.*, 1998; Porter *et al.*, 1998). BK_{Ca} channels have been shown to be involved in control of cell excitability in various types of smooth muscle such as human cerebral arteries (Wellman *et al.*, 2002), guinea pig urinary bladder (Heppner *et al.*, 1997) and guinea pig ureter (Burdyga & Wray, 2005).

BK_{Ca} channels can be blocked by charybdotoxin and iberiotoxin and by tetraethylammonium(TEA) in low concentration. Inhibition of the SR Ca^{2+} release by ryanodine or CPA can abolish both calcium sparks and STOCs, causing cell membrane depolarization (Nelson *et al.*, 1995).

STOCs, is responsible for repolarizing phase of the action potential and can contribute to the resting membrane potential (Imaizumi *et al.*, 1996; Heppner *et al.*, 1997). Activation of STOCs by Ca^{2+} sparks could hyperpolarize cell membrane and close the voltage-dependant Ca^{2+} channels (Bryden & Nelson, 1992; Knot & Nelson, 1998; Knot *et al.*, 1998). Unlike cardiac and skeletal muscle, in smooth muscle the Ca^{2+} sparks can indirectly modulate the membrane potential and cause smooth muscle relaxation via activation of the Ca^{2+} sparks/STOCs coupling mechanism. Recent study shows that in guinea pig ureter smooth muscle Ca^{2+} sparks/STOCs coupling mechanism is present and can act as a negative feedback mechanism to control the excitability of the ureteric smooth muscle. Ca^{2+} sparks/STOCs coupling mechanism plays an important role in setting the refractory period (Burdyga & Wray, 2005) and duration of the plateau component of the action potential (Borisova *et al.*, 2007).

In vascular smooth muscle, activation of PKC could inhibit BK_{Ca} channel (Minami *et al.*, 1993; Schubert *et al.*, 1999; Crozatier, 2006; Ledoux *et al.*, 2006). BK_{Ca} channel can be activated by some vasodilators via activation of PKC (Song & Simard, 1995; Standen & Quayle, 1998). The α-subunit of BK_{Ca} channel can be phosphorylated by cAMP-dependent PKA (Tian *et al.*, 2001; Zhou *et al.*, 2001) and PKC (Reinhart *et al.*, 1995). RACK, a scaffolding protein of PKC, is shown to be a BK_{Ca} channel binding protein (Isacson *et al.*, 2007). In cerebral arteries, activation of PKC decreased the frequency of both Ca^{2+} sparks and STOCs therefore inhibiting BK_{Ca} channel activity indirectly (Bonev *et al.*, 1997). Inhibition of PKC was also shown to inhibit Ca^{2+}

sparks in airway smooth muscle (Liu *et al.*, 2007).

Ca^{2+} sparks/STOCs coupling mechanism is known to play a negative feedback mechanism to control the smooth muscle excitability. Is Ca^{2+} sparks/STOCs coupling mechanism involved in the stimulant action of PKC in the guinea pig ureteric smooth muscle? To answer this question, we have investigated possible role of Ca^{2+} sparks/STOCs coupling mechanism in stimulant action of PDBu in the guinea pig ureteric smooth muscle. We have investigated the effects of PDBu on action potential, Ca^{2+} transients and force to prove that an increase in duration of the Ca^{2+} transient by PDBu was indeed associated with the prolongation of the plateau component of the action potential. Secondly we have investigated the effects of PDBu on Ca^{2+} transients and force evoked by EFS in the presence of TEA to block BK_{Ca} channels and CPA to block Ca^{2+} sparks. Thirdly, we have investigated the effects of PDBu on Ca^{2+} sparks using confocal imaging of guinea pig ureteric myocites.

4.2 Materials and Methods

Simultaneous measurement of calcium and Force

The simultaneous measurement of force and intracellular calcium were described in Chapter 2.

Confocal imaging of Fluo-4 loaded ureteric cells was performed using Ultra View Nipkow disc based imaging system.

Krebs solution was prepared as described in Chapter 2. The tissue was continuously perfused with Krebs, Krebs containing CPA (20μM), TEA (10mM), PDBu (0.1μM) and caffeine (1mM).

The ureter smooth muscle were stimulated electrically at interval of about 40 seconds to produce phasic contraction associate with Ca^{2+} transients by using Ag/AgCl electrodes placed in the bath with rectangular pulses (3-5V, 200ms).

Chemicals

All chemicals were purchased from Sigma (Dorset, UK), unless otherwise stated. PDBu was purchased from Calbiochem.

The stock solutions were prepared as following method: PDBu was dissolved in DMSO at a concentration of 1mM. CPA was dissolved in ethanol at a concentration of

20 mM. The stock solutions were diluted to the desired concentrations with Krebs solution before starting the experiment.

Statistics

Data was analysed with t-test; differences between means were assumed to be significant at $P<0.05$. One-Way ANOVA test was applied to test the significant difference ($P<0.05$) between different groups. All values represent mean ± s.e.m; *n* is the number of samples, each one from a different animal.

4.3 Results

4.3.1 Effects of PKC activator PDBu on action potential, calcium transients and phasic contractions in the guinea pig ureteric smooth muscle

Using double sucrose gap method combined with photometric measurement of $[Ca^{2+}]_i$ and force in Indo-1 loaded strips of the guinea pig ureter the effects of PDBu on the action potential, Ca^{2+} transients and force were recorded simultaneously. The ureteric strips were stimulated by short (100m sec) suprathershold depolarizing pulses. Figure 4.1 shows that PDBu produced prolongation of the plateau component of the action potential which was associated with an increase in the duration of the Ca^{2+} transient and the amplitude and duration of the phasic contraction. These data clearly indicate that changes in the duration of the Ca^{2+} transients and force produced by PDBu described in Chapter 3 were associated with an increase in the duration of the plateau component of the action potential. The increase in duration of the plateau component of the action potential can be produced by either inhibition of Ca^{2+} sparks or BK_{Ca} channels (Borisova *et al.*, 2007). Since both mechanisms were reported to be inhibited by PKC at least in some types of smooth muscle (see introduction of this chapter). We next investigated the effects of PDBu on Ca^{2+} transients and force evoked by EFS in the presence of TEA or CPA.

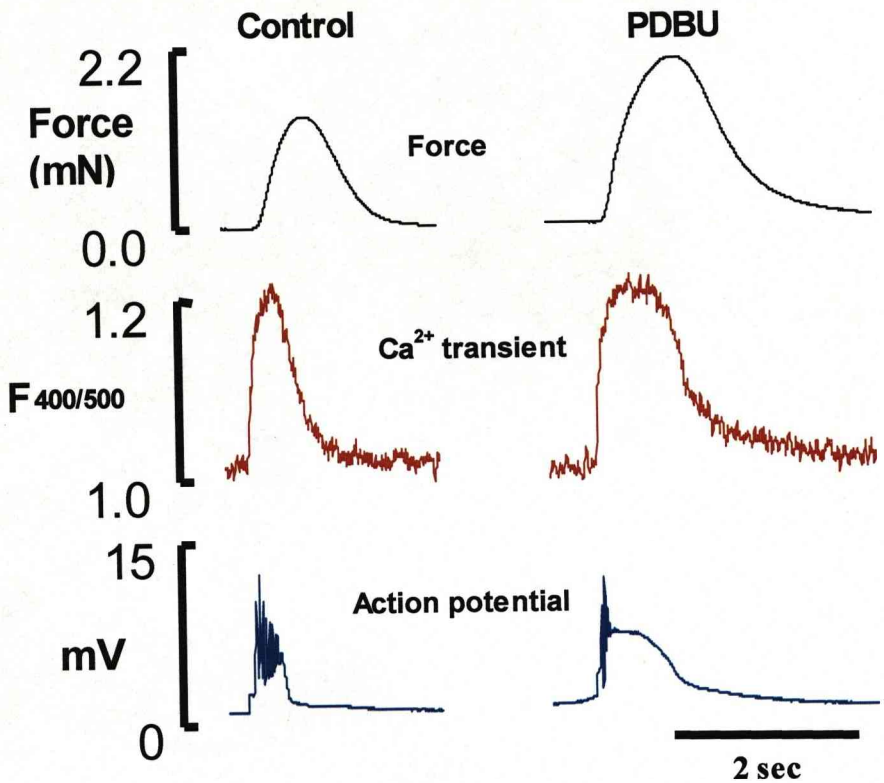


Figure 4.1 Simultaneous records of action potential (bottom trace), calcium transients (middle trace) and phasic contractions (top trace) in the absence and presence of PDBu (0.1μM). (Dr. T. Burdyga, unpublished data).

4.3.2 Effects of PDBu on calcium transients and phasic contractions evoked by EFS in the presence of TEA

Tetraethylammonium(TEA) at concentration of 10mM was used to block the Ca^{2+}-activated K^+ (BK_{Ca}) channels in guinea pig ureteric smooth muscle.

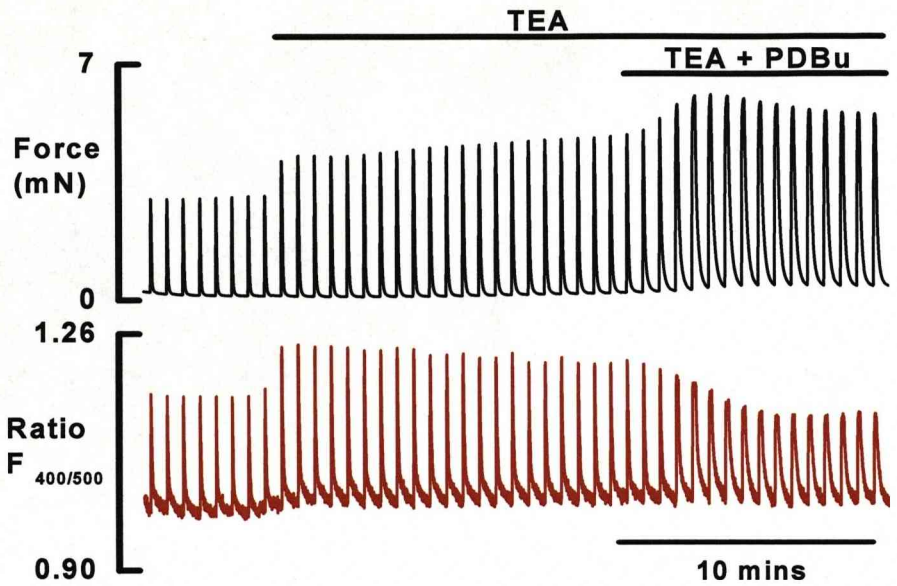
Figure 4.2 shows typical record of changes in Ca^{2+} transients and force evoked by EFS in the presence of TEA and combined action of TEA and PDBu. This figure shows that TEA produced an increase in the amplitude and duration of the phasic contraction which was mainly associated with an increase in the duration of the Ca^{2+} transient (Figure 4.3). PDBu produced marked increase in the amplitude but especially duration of the phasic contraction although the amplitude of the Ca^{2+} transients in the presence of TEA and PDBu was reduced (Figure 4.2 and 4.3). These data strongly suggest that stimulant action of PDBu in the guinea pig ureter smooth muscle does not involve inhibition of BK_{Ca} channels. In fact the data obtained suggest that the stimulant action of PDBu in the presence of TEA was potentiated.

Thus, PDBu increased the amplitude of force 1.9 ± 0.1 times and duration of force and Ca^{2+} transient in the presence of TEA 6.2 ± 0.1 times and 12.2 ± 0.06 times compared to control, respectively (n=10) ($P<0.05$). This increase in the duration of Ca^{2+} transient and force in the presence of TEA was associated with an increase in the

duration of the plateau component of the action potential (Figure 4.4).

These data collectively suggest that BK_{Ca} channels are not inhibited by PKC activation in the guinea pig ureteric smooth muscle.

A



B

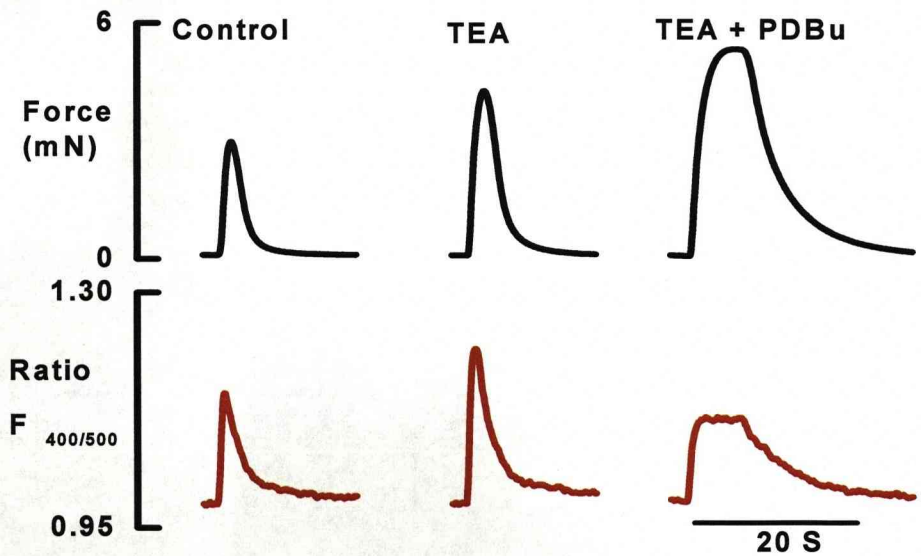


Figure 4.2 The effect of PDBu on Ca^{2+} and phasic contractions evoked by EFS in guinea pig ureter smooth muscle in the presence of TEA. (A) Original trace shows Ca^{2+} transients and phasic contraction recorded under control condition, in the presence of TEA and TEA with PDBu; (B) Extracted trace from (A) shows individual Ca^{2+} transients and phasic contraction in guinea pig ureter smooth muscle.

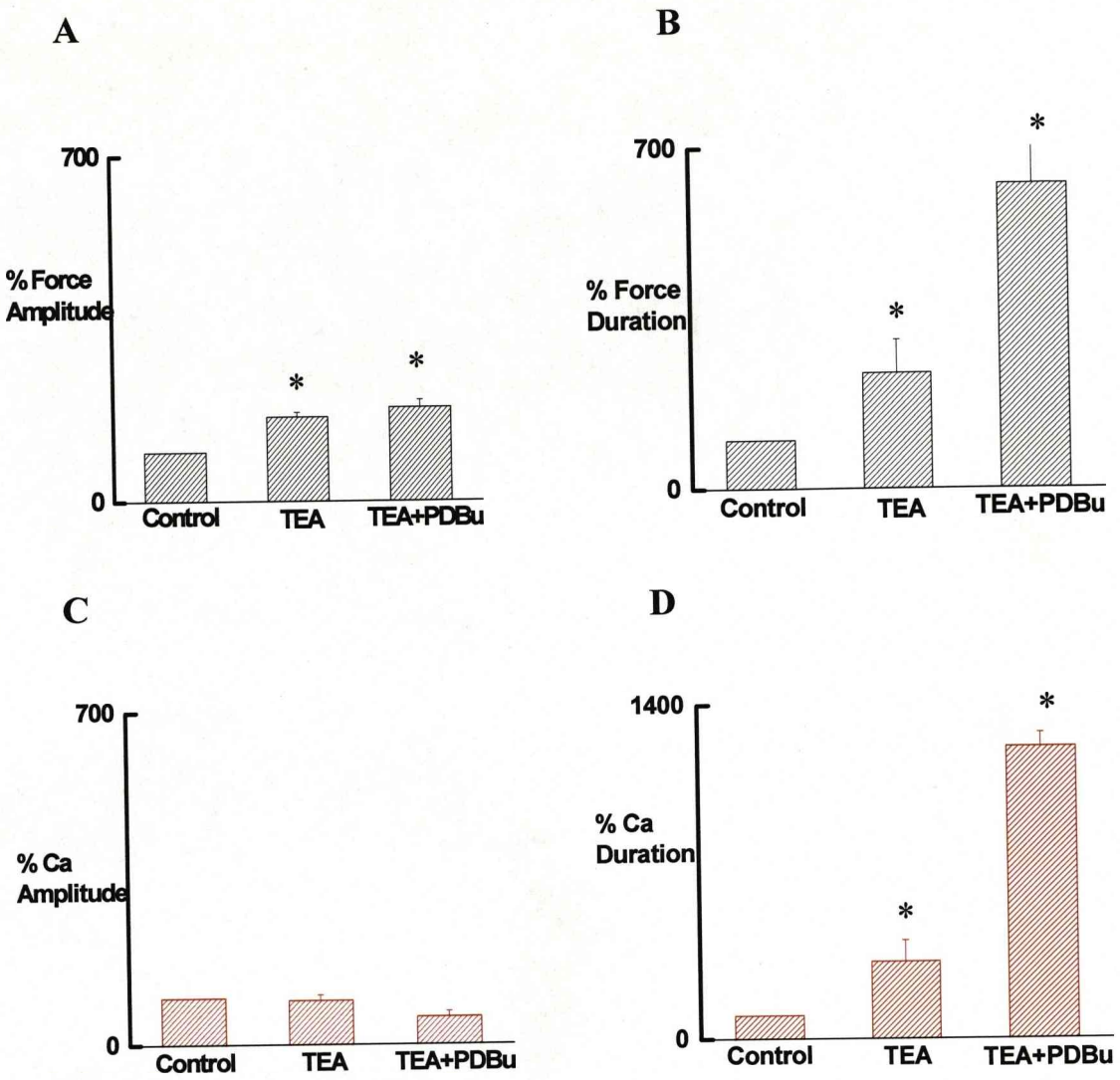


Figure 4.3 Mean values of amplitude (A) and duration (B) of force and amplitude (C) and duration (D) of Ca^{2+} transients in the absence and presence of TEA and in the presence of TEA with PDBu and expressed as percentage of control.

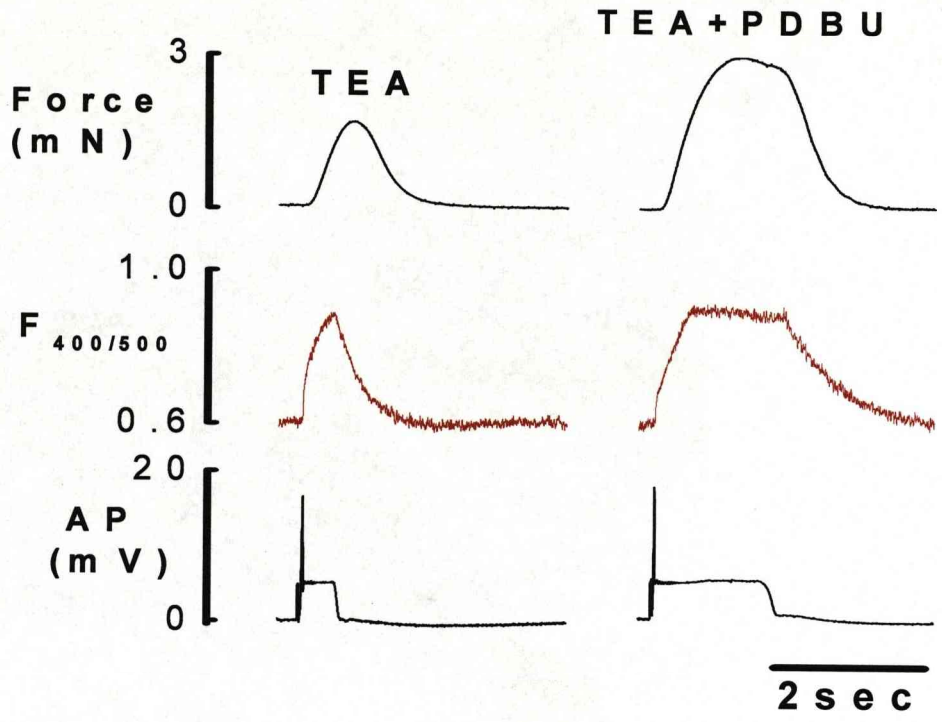


Figure 4.4 The effect of PDBu on the action potential, Ca^{2+} transients and phasic contraction in guinea pig ureter smooth muscle in the presence of TEA (10mM) and TEA with PDBu. (Dr. T. Burdyga, unpublished data).

4.3.3 Effects of PDBu on calcium transients and phasic contractions evoked by EFS in the presence of CPA

In this study, cyclopiazonic acid (CPA), a selective blocker of the SR Ca^{2+}-ATPase, was used to block the SR function and deplete the SR Ca^{2+} store to inhibit Ca^{2+} sparks. In previous studies, it was found that treatment of guinea pig ureter smooth muscle with CPA at concentration of 20 μM was optimal to completely deplete the SR and abolish Ca^{2+} sparks (Burdyga *et al.*, 1995).

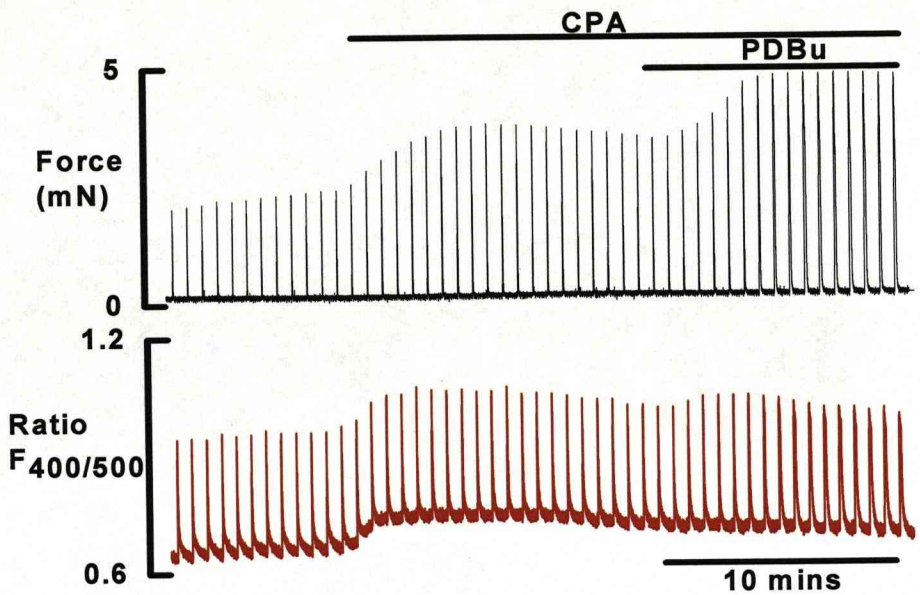
Figure 4.5 shows that CPA increased the baseline level of Ca^{2+} and an increase in the amplitude and duration of the force 2.3 ± 0.27 times and 1.5 ± 0.2 times (n=12) ($P<0.05$) compared to control, respectively. This increase in amplitude of force was accompanied by an increase in the duration of the Ca^{2+} transient 1.5 ± 0.2 times of control (n=12) ($P<0.05$) (Figure 4.6).

In the presence of CPA, PDBu still showed marked stimulant effect on both force and Ca^{2+} transient. In the presence of CPA, PDBu increased the amplitude and duration of the force which was accompanied by the increase in duration of the Ca^{2+} transients (Figure 4.5 and 4.6).

Thus, the data obtained in this study strongly suggest that an increase in duration of the Ca^{2+} transient by PDBu in the guinea pig ureteric smooth muscle was not

associated with possible inhibition of Ca^{2+} sparks. In fact, stimulant action of PDBu on the amplitude and especially duration of force in the presence of CPA were enhanced (Figure 4.5 and 4.6).

A



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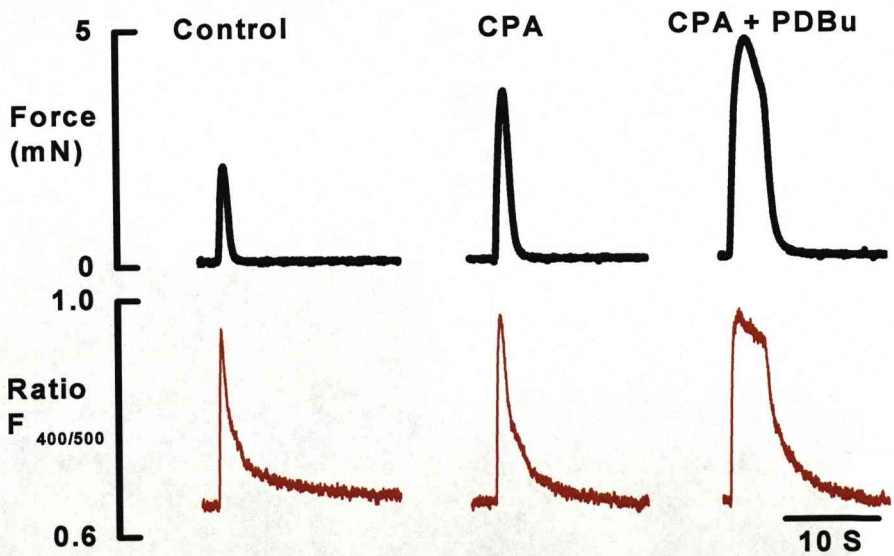


Figure 4.5 The effect of PDBu on Ca^{2+} transient and phasic contractions evoked by EFS in guinea pig ureter smooth muscle in the presence of CPA. (A) Original trace shows Ca^{2+} transients and phasic contraction recorded under control condition, in the presence of CPA and CPA with PDBu; (B) Extracted trace from (A) shows individual Ca^{2+} transients and phasic contraction in guinea pig ureter smooth muscle.

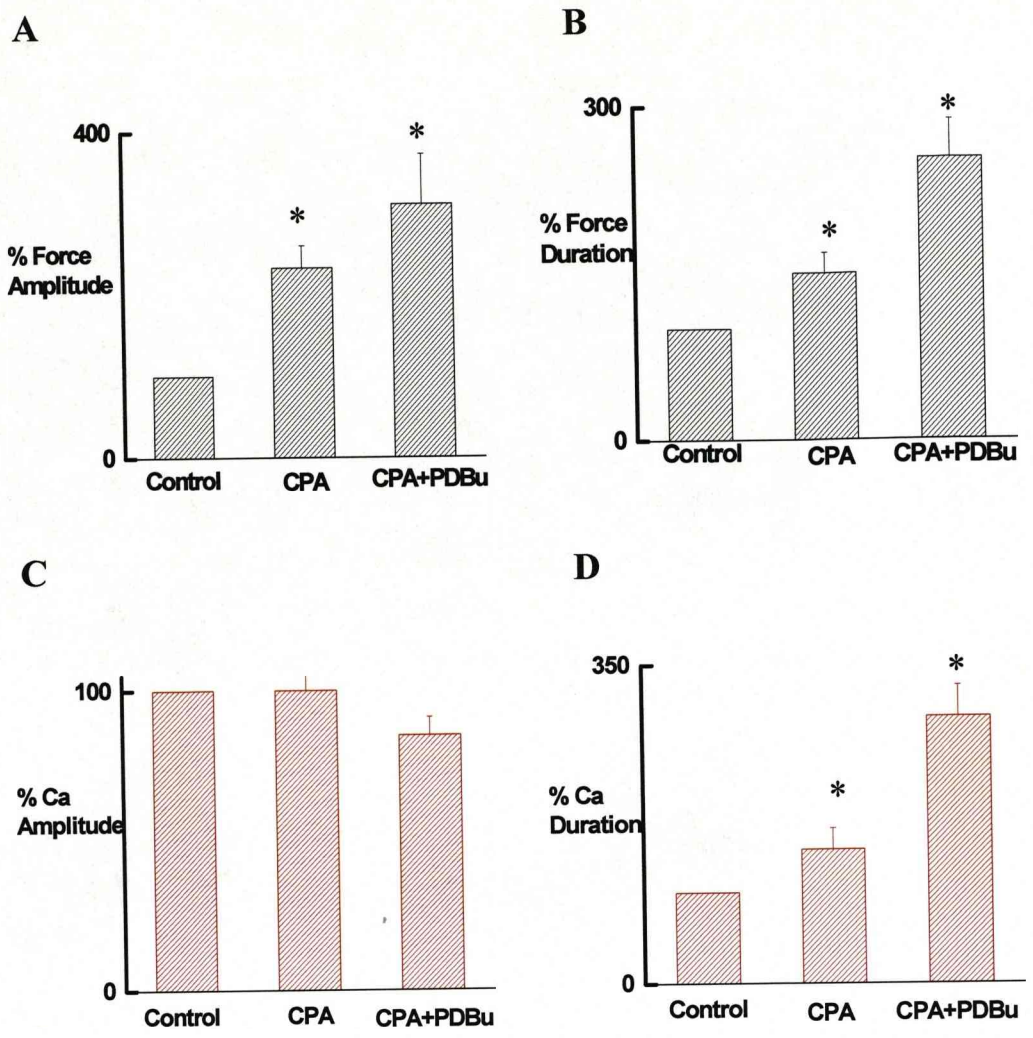


Figure 4.6 Mean values of amplitude (A) and duration (B) of force and amplitude (C) and duration (D) of Ca^{2+} transients in the absence and presence of CPA and in the presence of CPA with PDBu and expressed as percentage of control.

4.3.4 Effect of PDBu on Ca^{2+} sparks

In order to investigate whether PKC activation blocks Ca^{2+} sparks in the guinea pig ureteric myocytes, the effects of PDBu on spontaneous Ca^{2+} sparks of isolated ureteric smooth muscle cells have been investigated. We have found that PDBu when applied to ureteric cells showing the presence of spontaneous Ca^{2+} sparks was not abolished by PDBu. In fact, it can be seen from Figure 4.7, PDBu produced an increase in the amplitude and frequency of spontaneous Ca^{2+} sparks. Again, these data strongly suggest that stimulant action of PDBu in guinea pig ureteric smooth muscle was not associated with direct inhibition of Ca^{2+} sparks.

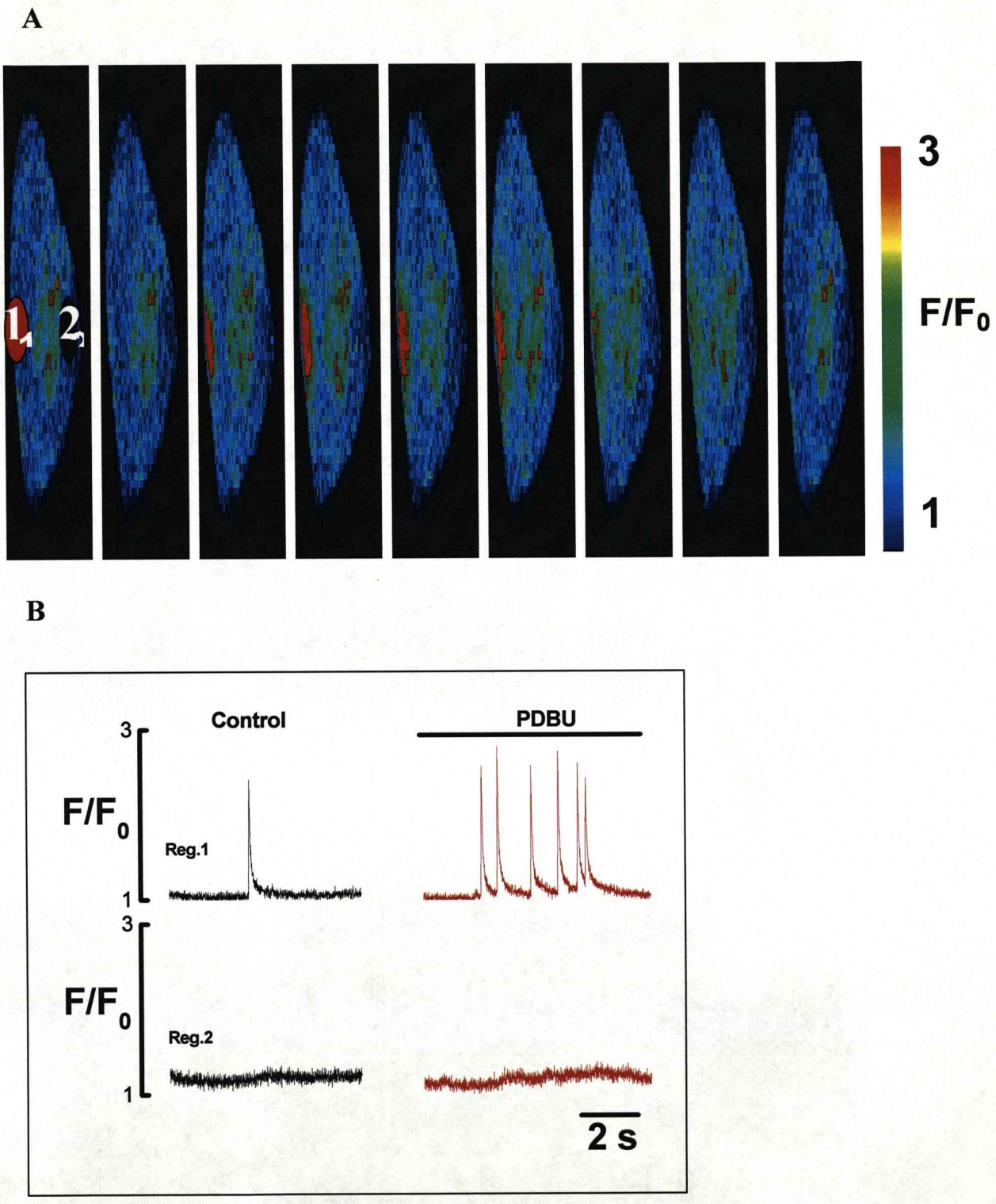


Figure 4.7. Effects of PDBu on spontaneous Ca^{2+} sparks in isolated guinea pig ureteric myocites. A- Stack of images showing sptemporal and spatial characteristics of spontaneous Ca^{2+} spark. B- changes in fluorescence signals in the discharging (Reg.1) and non-discharging (Reg.2) sites in the absence and presence of PDBu (10 minutes).

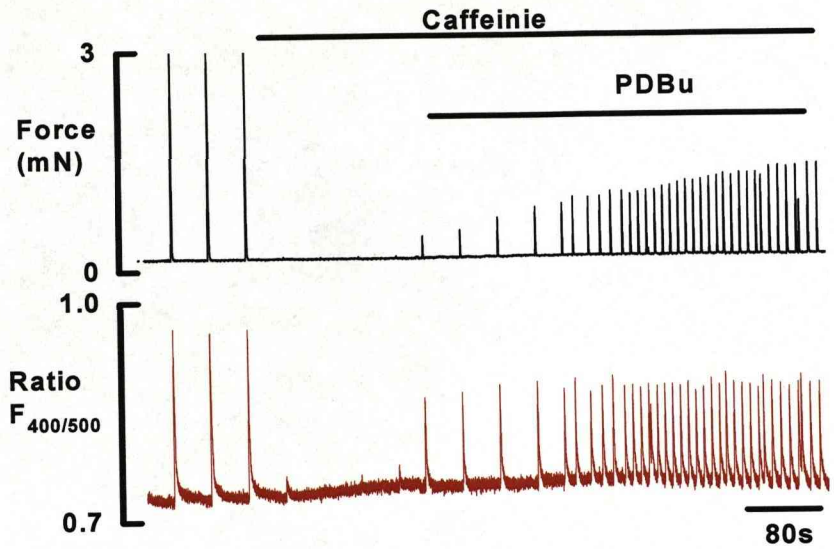
4.3.5 Effects of PDBu on calcium transients and phasic contractions evoked by EFS in the presence of caffeine

Caffeine can activate Ca^{2+} induced Ca^{2+} release (CICR) by sensitizing RyR to Ca^{2+}. It has been shown that in the guinea pig ureter caffeine at low concentration can increase Ca^{2+} sparks activity in smooth muscle to activate Ca^{2+} sparks/ STOCs coupling mechanism leading to inhibition of action potential (Borisova *et al.*, 2007).

Caffeine at low concentration (1mM) was shown to have no effect on either Ca^{2+} current or Ca^{2+} transients in voltage clamped ureteric myocytes (Borisova *et al.*, 2007). Caffeine (1mM) produced an inhibitory effect on force and Ca^{2+} transients evoked by EFS in the guinea pig ureteric smooth muscle (Figure 4.8 and 4.9).

Stimulant action of PDBu under conditions when Ca^{2+} sparks/STOCs coupling mechanism was maximized by caffeine was significantly decreased suggesting that activation of PKC in the guinea pig ureter does not involve inhibition of Ca^{2+} sparks/STOCs coupling mechanism.

A



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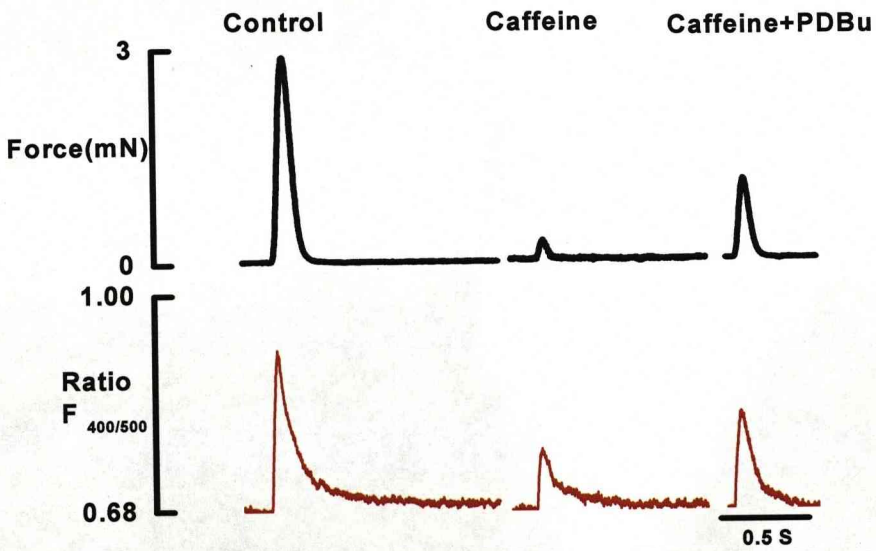


Figure 4.8 The effect of PDBu on Ca^{2+} transients and phasic contractions evoked by EFS in guinea pig ureter smooth muscle in the presence of caffeine (1μM). (A) Original trace shows Ca^{2+} transients and phasic contraction recorded under control condition, in the presence of caffeine and caffeine with PDBu; (B) Extracted trace from (A) shows individual Ca^{2+} transients and phasic contraction in guinea pig ureter smooth muscle.

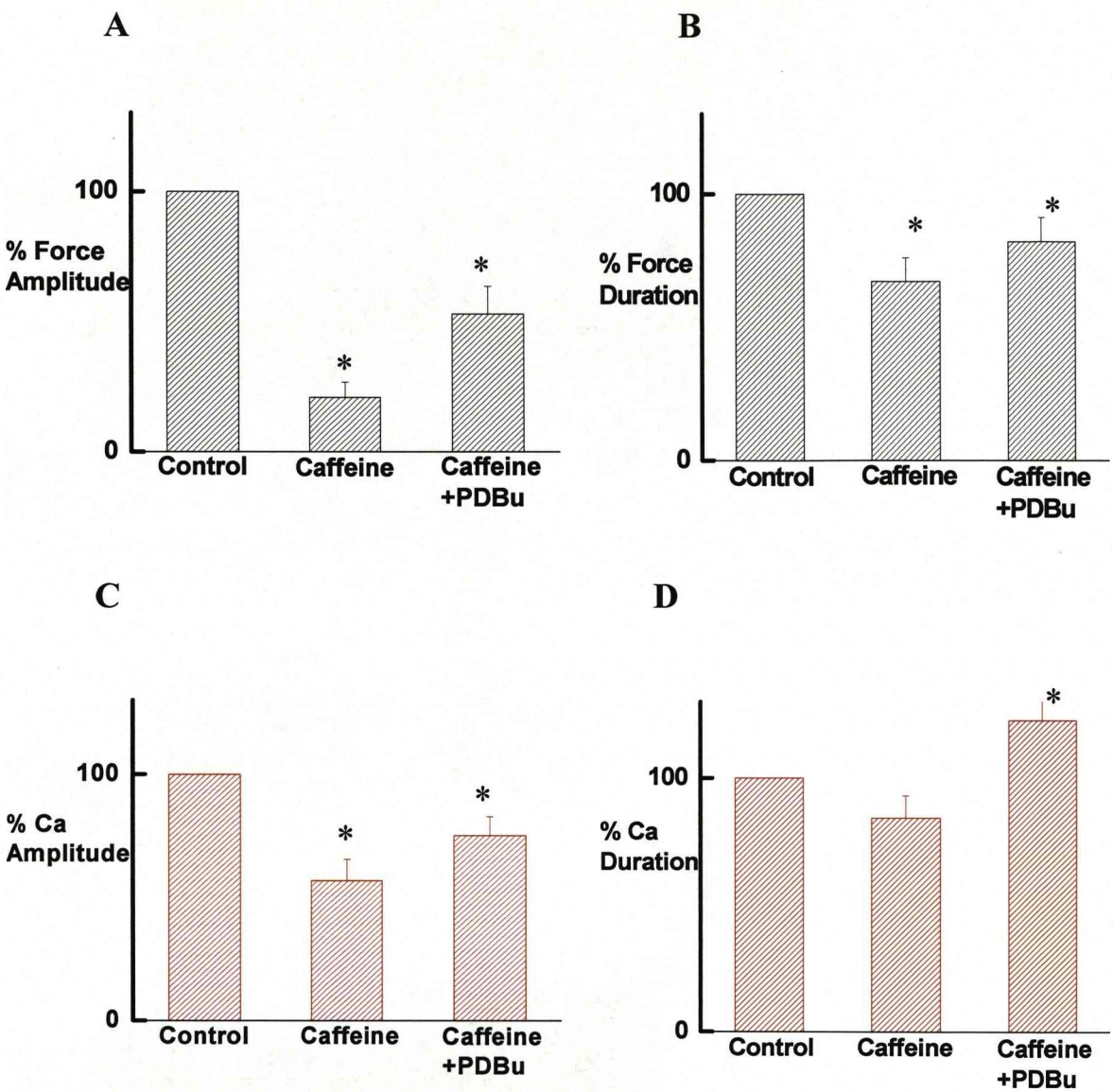


Figure 4.9 Mean values of amplitude (A) and duration (B) of force and amplitude (C) and duration (D) of Ca^{2+} transients in the absence and presence of caffeine and in the presence of caffeine with PDBu and expressed as percentage of control.

4.4 Discussion

PKC activator PDBu significantly prolonged the duration of the plateau component of action potential in guinea pig ureteric smooth muscle. This effect on the action potential can be achieved either by activation of L-type Ca^{2+} channels or inhibition of BK_{Ca} channels or combination of both.

Ca^{2+} sparks/ STOCs coupling mechanism plays a key role in control of refractory period in the guinea pig ureteric smooth muscle. If Ca^{2+} sparks or STOCs were blocked, the refractory period disappeared (Burdyga & Wray, 2005). In addition, Ca^{2+} sparks/ STOCs coupling mechanism plays an important role in control of the duration of the complex action potential in guinea pig ureteric smooth muscle (Burdyga & Wray, 1999; Borisova *et al.*, 2007). Inhibition of Ca^{2+} sparks or STOCs by CPA and TEA, respectively, produced stimulant effect on guinea pig ureteric smooth muscle by increasing the plateau component of the action potential. CPA is shown to completely disable the SR function therefore Ca^{2+} failed to load into SR resulting in the elevation of the intracellular Ca^{2+} concentration which could explain the elevation of the baseline of $[Ca^{2+}]_i$ produced by CPA in this study. In the present study we showed that in the presence of CPA or TEA PDBu still produced prominent stimulant effect on Ca^{2+} transients and phasic contractions evoked by EFS in the guinea pig ureteric smooth muscle.suggesting that Ca^{2+} sparks/STOCs coupling mechanism is unlikely to be involved in the stimulant action of PDBu. The data with TEA also suggest that

direct effects of PKC activation on BK_{Ca} channels are also unlikely.

Caffeine, as an activator of CICR, has been shown to decrease the duration of the plateau component of the action potential associated with maximal activation of Ca^{2+} sparks/STOCs coupling mechanism in the guinea pig ureter (Borisova *et al.*, 2007). This effect of caffeine in the guinea pig ureteric smooth muscle is in contrast to its stimulant effect seen in cardiac muscle (O'Neil *et al.*, 1990). In the presence of low concentration of caffeine, stimulant action of PDBu was diminished. suggesting that inhibitory effect of caffeine via Ca^{2+}sparks/STOCs coupling mechanism is not inhibited by activation of PKC.

The results obtained show that activation of PKC increased Ca^{2+} spark frequency in the guinea pig isolated ureteric smooth muscle cells. This may be due to the direct action of PKC on the RyRs (Takasago *et al.*, 1991). In cerebral arteries, activation of PKC decreased Ca^{2+} sparks frequency through a direct action on RyRs (Bonev *et al.*, 1997). However, in rabbit portal vein, activation of PKC inhibited BK_{Ca} channels directly but not via Ca^{2+} sparks (Kitamura *et al.*, 1992). In this study we showed that PDBu produced stimulant rather than inhibitory effects on Ca^{2+} sparks. Since in the guinea pig ureter smooth muscle, Ca^{2+} sparks activate BK_{Ca} channels, generating STOCs (Burdyga & Wray, 2005). Therefore, by exerting the stimulant action on Ca^{2+} sparks, PDBu could be able to increase the BK_{Ca} channels activity therefore enhancing STOCs and Ca^{2+} sparks/STOCs coupling mechanism. One could expect

that PDBu could have produced inhibitory rather than stimulant effects on Ca^{2+} transients and force. Stimulant effect of PDBu on Ca^{2+} sparks activity could be expected to decrease the duration of action potential and Ca^{2+} transient. The fact that stimulant action of PDBu on duration of Ca^{2+} transient in the presence of either TEA or CPA was stronger comparing to control conditions suggests that PDBu could indeed stimulate Ca^{2+}sparks/STOCs coupling mechanism along with much stronger effects on the inward currents which results in the overall stimulant action.

In conclusion, we are the first to provide the evidence that activation of PKC increases Ca^{2+} sparks frequency in guinea pig ureteric smooth muscle. PKC activator PDBu could increase the amplitude and duration of force and Ca^{2+} in the presence of CPA and TEA, which could be explained that activation of PKC has no inhibitory effect on the Ca^{2+} sparks/STOCs coupling mechanism.

Chapter 5

PKC activation and Na^+-dependent mechanisms in guinea pig ureter

Chapter 5

PKC activation and Na^+-dependent mechanisms in guinea pig ureter

5.1 Introduction

Na^+ has been shown to play an important role in control of smooth muscle excitation-contraction coupling (Branding *et al.*, 1990). The regulation of Na^+ in smooth muscle cells can involve Na^+-K^+ pump or Na^+-Ca^{2+} exchanger. Currently, there is no direct evidence of inward Na^+ current in guinea pig ureter smooth muscle. In addition, application of tetrodotoxin (TTX) which is a potent inhibitor of voltage-dependent Na^+ channel in other muscle tissues, does not have effect on either inward currents or action potential in guinea pig ureter (Kuriyama *et al.*, 1967; Sui *et al.*, 1997). Therefore, it is suggested that there is no TTX-sensitive voltage gated Na^+ channel in this tissue. Furthermore, removal of extracellular Na^+ has no effect on currents carried by other ions (Sui *et al.*, 1997).

In guinea pig ureter, the action potential is known to consist of a spike component and a long lasting plateau component. Both Na^+ and Ca^{2+} can contribute to maintain the duration of the plateau component of action potential (Shuba, 1977(2)). It is known that Ca^{2+} carries predominantly the inward current in this tissue. No inward Na^+ current was found in guinea pig ureteric myocites (Sui *et al.*, 1997). The evidence for Na^+-Ca^{2+} exchanger has been obtained for guinea pig ureter (Aickin *et al.*, 1984;

1987). Normally, Na^+-Ca^{2+} exchanger extrudes one Ca^{2+} out of cell in exchange of three Na^+ into the cell. Therefore, it is electrogenic and can affect the membrane potential. The direction of movement of these ions depends upon the membrane potential and the chemical gradient for Ca^{2+} and Na^+. Under certain condition, for example, after inhibition of Na^+-K^+ pump by ouabain, the exchanger begins to operate in "Ca^{2+} entry mode" (Aickin *et al.*, 1984). However, there is evidence that Na^+-Ca^{2+} exchanger can also operate in normal physiological conditions. Aaronson *et al.* has shown that Na^+-Ca^{2+} exchanger in single voltage clamped guinea pig ureteric myocyte can be involved in control of Ca^{2+} signaling in physiological range of $[Na^+]_i$ (Aaronson *et al.*, 1989). In addition, by using the SBFI- fluorescent Na^+-sensitive indicator to measure $[Na^+]_i$, Lamont *et al.* provided further evidence of Na^+-Ca^{2+} exchanger and its role in modulating the force contraction in guinea pig ureter (Lamont *et al.*, 1998). In accordance with literature, PKC can modulate activity of Na^+-Ca^{2+} exchanger. In vascular smooth muscle cells of conduit arteries such as aorta (Mashiburn *et al.*, 1997) and small renal arterioles (Khoyi *et al.*, 1991) stimulant action of PKC activation was associated with activation of the Na^+-Ca^{2+} exchanger (Fowler *et al.*, 1996). Therefore in the next series of experiments we have studied possible role of Na^+-Ca^{2+} exchanger in the stimulant action of PDBu in the guinea pig ureter. This is achieved by measuring force and calcium simultaneously induced by activation of the Na^+-Ca^{2+} exchanger in "Ca^{2+} entry" mode in Na^+ loaded guinea pig ureteric muscle as described earlier (Aickin *et al.*, 1984; Lamount *et al.*, 1998).

5.2 Materials and Methods

Simultaneous measurement of calcium and Force

The measurement of force and intracellular calcium were described in Chapter 2.

Krebs solution was prepared as described in Chapter 2. The tissue was continuously perfused with Krebs solution. Ouabain (10^{-4} M) was used to inhibit Na^+-K^+ pump for 1 hour to produce Na^+ loading ureteric tissue. Na^+-free (Tris substitution) was used to stimulate the ureteric strips loaded with Na^+ to activate Na^+-Ca^{2+} exchanger in Ca^{2+} entry mode.

The ureteric smooth muscle was stimulated electrically at interval of about 40 seconds to produce phasic contraction associate with Ca^{2+} transients by using Ag/AgCl electrodes placed in the bath with rectangular pulses (3-5V, 200ms).

Chemicals

All chemicals were purchased from Sigma (Dorset, UK), unless otherwise stated. PDBu was purchased from Calbiochem.

The stock solutions were prepared as following method: PDBu was dissolved in DMSO at a concentration of 1mM. The stock solutions were diluted to the desired concentrations with Krebs solution before starting the experiment.

Statistics

Data was analysed with t-test; differences between means were assumed to be significant at $P<0.05$. One-Way ANOVA test was applied to test the significant difference ($P<0.05$) between different groups. All values represent mean ± s.e.m; *n* is the number of samples, each one from a different animal.

5.3 Results

5.3.1 Effects of PDBu on force and calcium evoked by EFS in the absence of extracellular Na^+

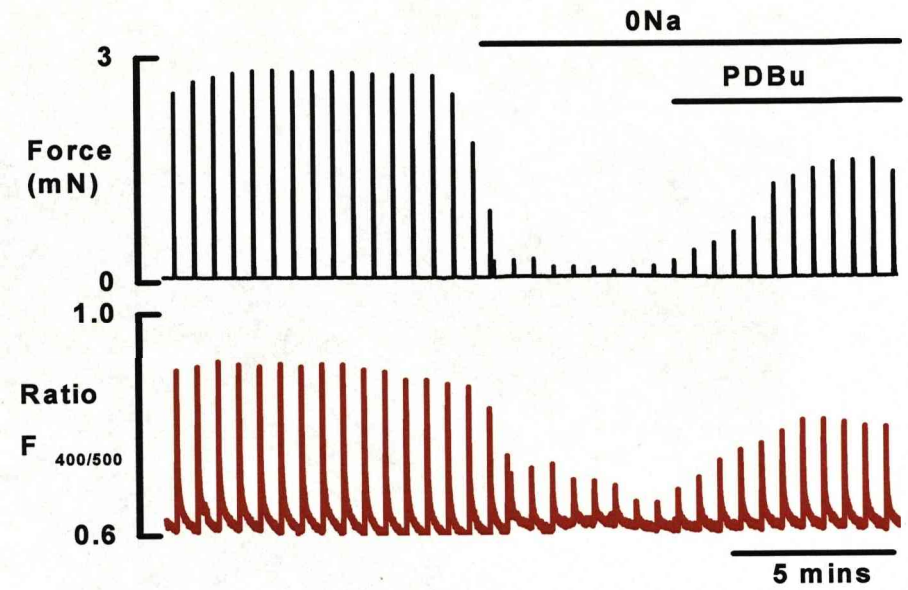
Removal of extracellular Na^+ shortened the duration of plateau component of the action potential in guinea pig ureter smooth muscle (Shuba, 1977 (2)). Hence, it was suggested that the extracellular Na^+ is important for generation of the plateau component of the action potential. Therefore, the effects of PKC activator PDBu on Ca^{2+} and force evoked by EFS in the guinea pig ureter smooth muscle in Na^+ free solution were investigated next.

In this experiments ureteric strips were stimulated by electrical field stimulation at about 40 seconds intervals to obtain a regular pattern of phasic contractions associated with Ca^{2+} transients. Removal of Na^+ from the bathing solution produced a rapid inhibitory effect on both force and Ca^{2+} in guinea pig ureter within 1 minute (Figure 5.1). Addition of PDBu (0.1μM) to Na^+-free solution produced partial restoration of Ca^{2+} and force (Figure 5.1 A). As can be seen from Figure 5.1 B and Figure 5.2, in Na^+ free solution, the amplitude of Ca^{2+} transients and force was significantly reduced (n=7). PDBu when applied in Na^+-free solution produced significant restoration of the amplitude of both Ca^{2+} transients and force (Figure 5.1 and 5.2).

The data obtained clearly show that PDBu still has strong stimulant effect on Ca^{2+} and

force in the absence of extracellular Na^+ suggesting that Na^+ sensitive mechanisms controlling the duration of the plateau component of action potential are not involved in the stimulant action of PKC activation in the guinea pig ureter smooth muscle.

A



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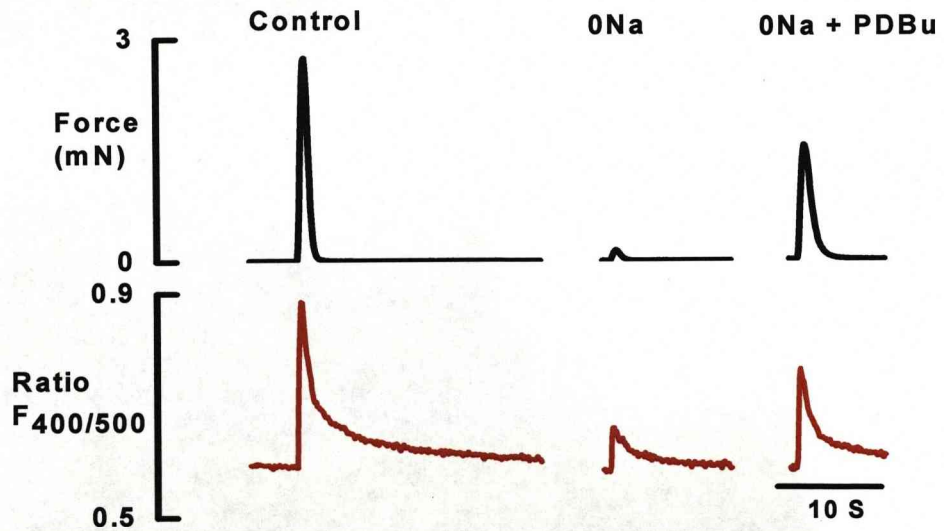


Figure 5.1 The effect of PDBu on Ca^{2+} transients and phasic contractions evoked by EFS in Na^{+}-free solution in the guinea pig ureter smooth muscle. A- Ca^{2+} transients and phasic contraction evoked by EFS in normal Krebs, Na^{+} -free solution and PDBu (0.1μM) applied in Na^{+} -free solution. B- Extracted single traces of Ca^{2+} transients and force recorded under control condition, in Na^{+}-free solution and in the presence of PDBu added to Na^{+}-free solution.

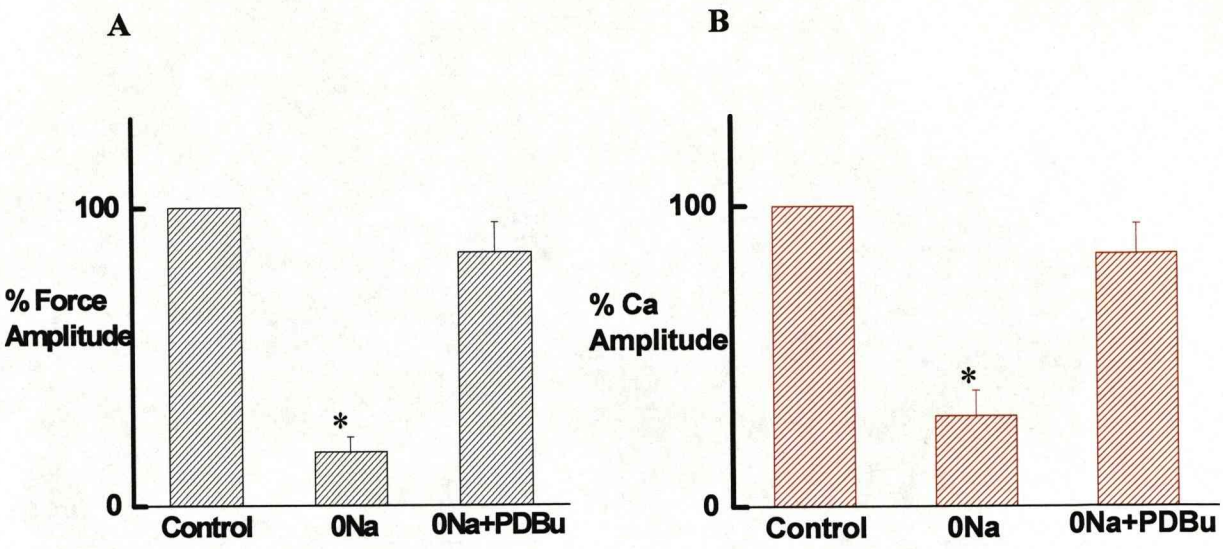


Figure 5.2 Restoration of the amplitude of force (A) and Ca^{2+} transients (B) by PDBu applied in Na^{+}-free solution. The amplitude of force and Ca^{2+} transients under control conditions were taken for 100%.

5.3.2 Effects of PDBu on the force and Ca^{2+} induced by Na^+-free solution in the Na^+-loaded guinea pig ureter smooth muscle

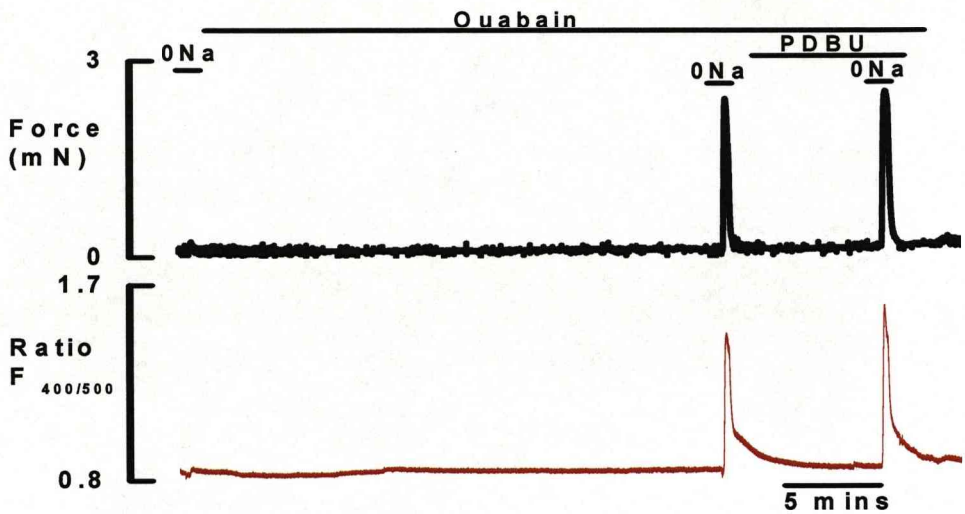
To investigate if Na^+-Ca^{2+} exchanger activity in guinea pig ureter smooth muscle is modulated by PKC, the effect of PKC activator PDBu on the Ca^{2+} and force induced by Na^+-free solution in the Na^+-loaded guinea pig ureter smooth muscle has been studied.

As was found previously (Aickin *et al.*, 1984), in normal ureteric tissue, removal of extracellular Na^+ had no effect on Ca^{2+} and force in guinea pig ureter smooth muscle (Figure 5.3 A). However, if the tissue was pretreated with Na^+-K^+ pump inhibitor ouabain (10^{-4}M) for 1 hour which enabled ureter to gain Na^+ (Aickin *et al.*, 1984). Na^+-loaded ureter when exposed to Na^+-free solution for a short time (40s) produced a rapid rise in Ca^{2+} and force activated by the reversed mode of Na^+-Ca^{2+} exchanger (Figure 5.3).

Application of PKC activator PDBu for 10 minutes had little effect on the amplitude of Ca^{2+} and force induced by Na^+-free solution (Figure 5.3). The extracted trace Figure 5.3 B from the original trace Figure 5.3 A clearly shows that PDBu had little effect on the amplitude of force which was only increased 1.04 ± 0.04 times (n=9) ($P>0.05$) compared to control (Figure 5.4 A). It also produced little changes in the amplitude of Ca^{2+} transient (1.02 ± 0.04 times, n=9) ($P>0.05$) (Figure 5.4 B).

Statistical analysis of the data suggests that Na^+-Ca^{2+} exchanger in at least Ca^{2+} entry mode is virtually insensitive to PKC activation. These data suggest prolongation of the duration of Ca^{2+} transient associated with the action potential produced by PKC activator PDBu does not involve Na^+-Ca^{2+} exchanger.

A



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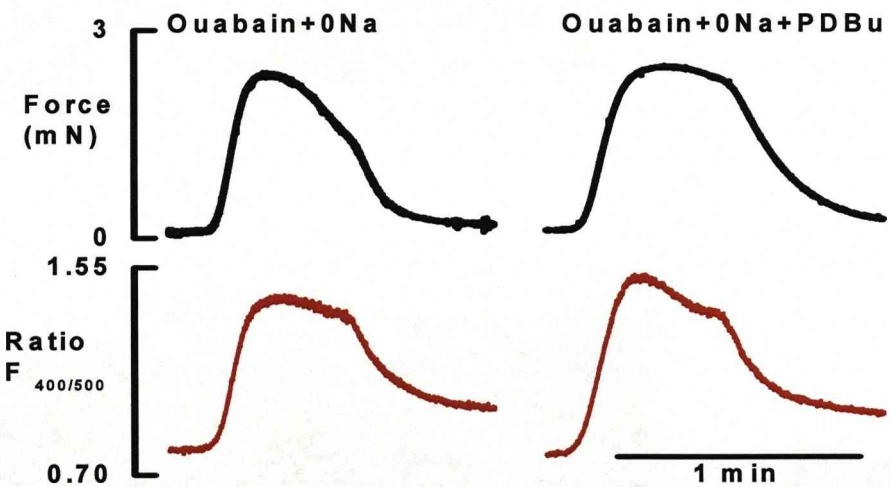


Figure 5.3 The effect of PDBu on Ca^{2+} transients and phasic contraction evoked by Na^{+}-free solution in Na^{+} -loaded guinea pig ureter smooth muscle. A- Original trace showing changes in $[Ca^{2+}]_i$ and force induced by Na^{+}-free solution (tris substitution) applied for 40s before and after treatment with ouabain (10^{-4} M) and their modulation by PDBu. B- Extracted data from A showing the individual Ca^{2+} transients and force induced by Na^{+}-free solution and Na^{+}-free solution with PDBu in the presence of ouabain.

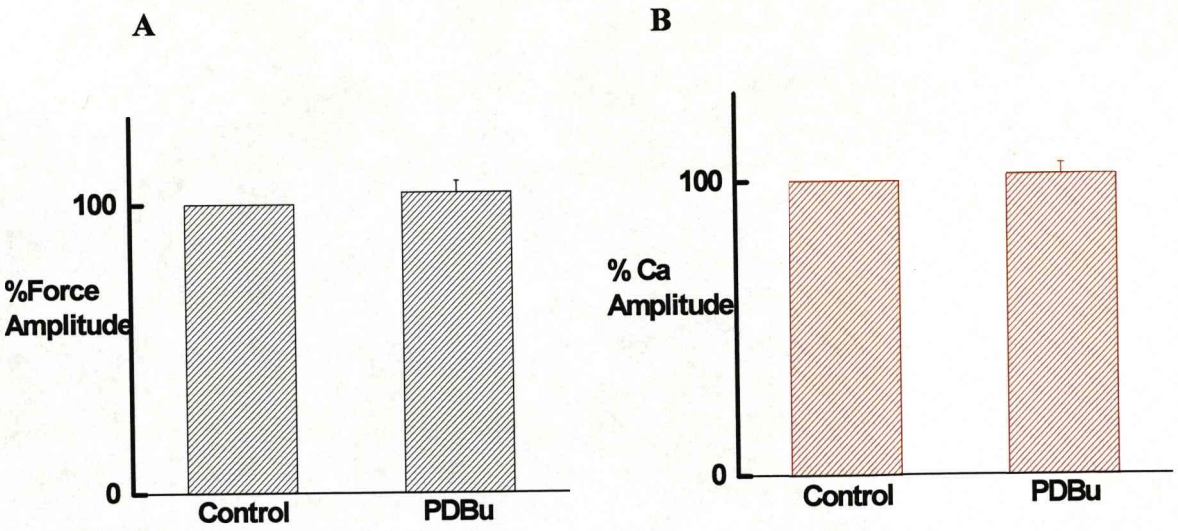


Figure 5.4 Mean values of amplitude of force (A) and Ca^{2+} transients (B) induced by Na^{+}-free solution (Control) and Na^{+}-free solution with PDBu for 10 minutes and expressed as percentage of control. Note lack of significant effect of PKC activation on the activity of Na^{+}- Ca^{2+} exchanger in "Ca^{2+}-entry" mode.

5.4 Discussion

Either removal of external Na^+ or application of Ca^{2+} antagonists (at low concentration) can lead to significant decrease in duration of the plateau component of the action potential in guinea pig ureter smooth muscle (Shuba, 1977(2)). Therefore, both extracellular Na^+ and Ca^{2+} are essential for maintaining the plateau component of action potential. Ca^{2+} can enter into the smooth muscle cells via L-type Ca^{2+} channel. However, it is not known how extracellular Na^+ is affecting parameters of the action potential in ureter smooth muscle. Since there is no voltage-dependent Na^+ channel found in this tissue, one of the possible assumptions is that both Ca^{2+} and Na^+ contribute to the generation of the plateau component of action potential via a common channel (Shuba, 1977(2)). Papaverine was shown to specifically block the common channel for both Ca^{2+} and Na^+ (Brading *et al.*, 1983). Since all the inward current can be abolished by Ca^{2+} channel blockers, another possible explanation is the findings of an extracellular Na^+ dependent late inward current which can be activated by elevation of $[Ca^{2+}]_i$ (Imaizumi *et al.*, 1989). The Na^+-Ca^{2+} exchanger might be the possible explanation for generating the late inward current. Meanwhile, Aaronson *et al.* also reported that Na^+-Ca^{2+} exchanger can modulate $[Ca^{2+}]_i$ in physiological normal conditions (Aaronson *et al.*, 1989). Nifedipine-resistant slow rises in $[Ca^{2+}]_i$ elicited by depolarization can be potentiated by reduction of the $[Na^+]_o$ or elevation of $[Ca^{2+}]_o$ have been reported for voltage clamped ureteric myocytes (Aaronson *et al.*, 1989). Other possibility which includes inactivation of Ca^{2+} current by extracellular

Na^+ as was found in cardiac muscle can not be excluded (Fedida *et al.*, 1987).

Previous work showed the evidence that Na^+-K^+ pump can be fully blocked by ouabain at a concentration of 10^{-4} M (Aickin, 1987(2)). After application of ouabain, either removal of extracellular Na^+ or elevation of extracellular Ca^{2+} can contribute to the extrusion of Na^+ in Na^+-loaded ureter producing a contraction (Aickin, 1987(1); Lamont *et al.*, 1998).

Na^+-Ca^{2+} exchanger can be studied by alteration of the Na^+ gradient across the cell membrane. This can be done either by removal of external Na^+ or an increase in internal Na^+ concentrations. In this work, the tissue with normal $[Na^+]_i$ failed to contract in response to Na^+-free solution. However, after inhibition of Na^+-K^+ pump with ouabain, $[Na^+]_i$ rised from the resting level at about 10mM to a level of 18-25mM reported by Lamont *et al.* (1998) and to a level of 20.6 mM reported by Aickin (Aickin, 1987(2)). Thus, Na^+-Ca^{2+} exchanger is suggested to be involved in this process to help maintain the low $[Na^+]_i$ level in the absence of Na^+-K^+ pump activity. However, the tissue maintains a relatively high level of intracellular Na^+ compared to control condition which can be rapidly lowered by a decrease in external Na^+ or an increase in external Ca^{2+} by Na^+-Ca^{2+} exchanger in reverse mode associated with entry of sufficient Ca^{2+} to initiate a contraction (Aickin *et al.*, 1984). This contraction is insensitive to Ca^{2+} antagonists and is associated with the loss of intracellular Na^+ (Aickin *et al.*, 1984). PKC activator PDBu does not significantly

increase the amplitude of Ca^{2+} and force induced by Na^{+}-free solution in Na^{+}-loaded ureter, which suggests that PKC is not involved in the regulation of Na^{+}-Ca^{2+} exchanger activity in its Ca^{2+} entry mode.

The amplitude of Ca^{2+} and force evoked by EFS was significantly decreased by removal of extracellular Na^{+}. This is consistent with the previous findings that removal or replacement of extracellular Na^{+} abolished the duration of the plateau component of action potential, only the initial spike component was seen (Shuba, 1977 (2); Brading *et al.*, 1983; Imaizumi *et al.*, 1989; Burdyga & Wray, 1999). PKC activator PDBu could still produce strong stimulant effect in Na^{+}-free solution. This suggests that the stimulant effects on Ca^{2+} and force by PDBu may not be associated with the extracellular Na^{+} and not with the activity of Na^{+}-Ca^{2+} exchanger in guinea pig ureter.

On the basis of the observations, the conclusion can be drawn that the influx of Na^{+} into the cells are important for excitation-contraction coupling mechanism in ureter smooth muscle, which is in general agreement with previous findings (Brading *et al.*, 1983; Shuba, 1977(2)). Moreover, the stimulant action of PDBu in Na^{+}-free solution is not abolished. Thus these data and the lack of effects of PDBu on the activity of Na^{+}-Ca^{2+} exchanger directly at least in Ca^{2+}-entry mode strongly suggest that the Na^{+}-Ca^{2+} exchanger or any other Na^{+}-dependent mechanism are not targeted by PKC in the guinea pig ureter and thus can not explain possible mechanism of the stimulant

action of PKC activation on duration of action potential and Ca^{2+} transient.

Chapter 6

Role of PKC in stimulant action of agonists in the guinea pig ureter smooth muscle

Chapter 6

Role of PKC in stimulant action of agonists in the guinea pig ureter smooth muscle

6.1 Introduction

In the guinea pig ureter smooth muscle, agonists such as carbachol and histamine produced stimulant action by increasing the duration of the plateau component of the action potential (Shuba, 1977(1); Burdyga & Wray, 1999).

Histamine, a biogenic amine involved in immune responses, acts directly on smooth muscles as a powerful stimulant agent. It may cause vasodilation, separation of endothelial cells and an increase in vascular permeability. Histamine or a related agent may play a physiological role in normal ureteric activity (Borgstedt *et al.*, 1962). By using double sucrose-gap method, Shuba showed that histamine prolonged the duration of the plateau of the action potential and increased the amplitude and duration of the phasic contraction (Shuba, 1977(1)). Histamine depolarized the cell membrane and initiated spontaneous action potential. The amplitude of the plateau component was not changed and the spike amplitude and duration were unaffected. The changes caused by histamine were relatively easily reversible. Histamine was shown to cause PKC translocation from cytosol to cell membrane in smooth muscle cells (Langlands *et al.*, 1992; Boterman *et al.*, 2005; Jin *et al.*, 2008). It was shown that histamine could activate latent pacemakers of the ureter's middle area (Kazarian

et al., 2003). Histamine exerts its function by binding to histamine receptors. There are four types of histamine receptors – H_1, H_2, H_3 and H_4, of which, H_1 histamine receptor was found in most types of smooth muscle and endothelium.

Adrenergic stimulation also produced stimulant action in the guinea pig ureter smooth muscle by increasing the duration of the plateau component of the action potential (Shuba, 1977(1)). In some vascular smooth muslce, the phenylephrine increased contraction at constant intracellular free Ca^{2+} concentration which is thought due to activation of a Ca^{2+}-independent isoform of PKC (Collins *et al.*, 1992). In human prostatic smooth muscle cells, phenylephrine can activate BK_{Ca} channel because this type of smooth muscle requires higher concentration of phenylephrine for contraction (Kurokawa *et al.*, 1998). It has also been shown that PKC is involved in phenylephrine-induced contraction in smooth muscle (Andrea & Walsh, 1992).

Therefore, the present study was undertaken to investigate the effect of exogenous agonists histamine and phenylephrine on force and Ca^{2+} evoked by EFS and to evaluate possible role of PKC involved in the stimulant action of these agonists.

6.2 Materials and Methods

Simultaneous measurement of calcium and Force

The simultaneous measurement of force and intracellular calcium were described in Chapter 2.

Krebs solution was prepared as described in Chapter 2. The tissue was continuously perfused with Krebs, Krebs containing histamine(1μM), phenylephrine(1μM) and Ro320432 (5μM).

The ureter smooth muscle were stimulated electrically at interval of about 40 seconds to produce phasic contraction associate with Ca^{2+} transients by using Ag/AgCl electrodes placed in the bath with rectangular pulses (3-5V, 200ms).

Chemicals

All chemicals were purchased from Sigma (Dorset, UK), unless otherwise stated. Ro320432 was purchased from Calbiochem.

The stock solutions were prepared as following method: Ro320432 was dissolved in DMSO at a concentration of 5mM. Histamine was dissolved in water at a concentration of 1mM. Phenylephrine was dissolved in water at a concentration of 1mM. The stock solutions were diluted to the desired concentrations with Krebs solution before starting the experiment.

Statistics

Data was analysed with t-test (paired or unimpaired); differences between means were assumed to be significant at $P<0.05$. All values represent mean ± s.e.m; *n* is the number of samples, each one from a different animal.

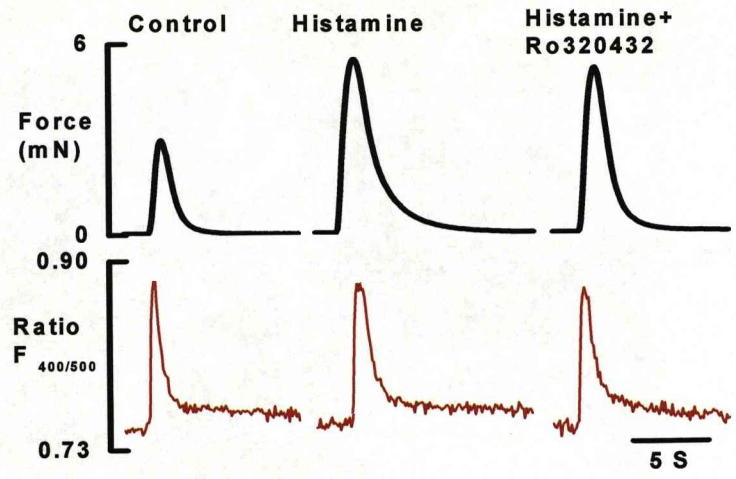
6.3 Results

Effects of PKC inhibitor Ro320432 on calcium transients and phasic contractions evoked by EFS in the guinea pig ureter smooth muscle in the presence of histamine and phenylephrine.

To determine if PKC is involved in the stimulant action of histamine and phenylephrine in the guinea pig ureter smooth muscle, we have studied the effects of PKC inhibitor Ro320432 on the stimulant effects of either histamine or phenylephrine on the amplitude and duration of the Ca^{2+} transients and force evoked by EFS. The experiments were performed on 14 animals for each of the agonists and presented in Figures 6.1 and 6.2, respectively. Figure 6.1 shows the representative trace of simultaneous recording of phasic contraction and Ca^{2+} transients in the guinea pig ureter evoked by EFS in control condition, in the presence of histamine (1μM) and in the presence of histamine (1μM) with Ro320432 (5μM). Figure 6.2 shows similar data for phenylephrine (1μM). From Figures 6.1 and 6.2, it can be seen that either of the agonists increased the amplitude of force evoked by EFS which was associated with an increase in the duration of the Ca^{2+} transient (Figure 6.1 B for histamine and Figure 6.2 B for phenylephrine). Treatment of the ureteric strips with PKC inhibitor Ro320432 for 20 minutes produced substantial decrease in the stimulant action of histamine (Figure 6.1) and phenylephrine (Figure 6.2) mainly on the duration of the Ca^{2+} transient and force. Therefore, the duration of the Ca^{2+} transient in the absence and presence of histamine was 0.75 ± 0.04 s and 1.38 ± 0.07 s, respectively ($P<0.05$).

After treatment of Ro320432 it was reduced to 0.92 ± 0.04 s. The duration of the Ca^{2+} transient in control conditions, presence of phenylephrine and combined action of phenylephrine and Ro320432 were 0.63 ± 0.05 s, 0.93 ± 0.12 s and 0.76 ± 0.02 s ($P<0.05$), respectively. Changes in the amplitude and the durations of force and Ca^{2+} expressed as percentage of control during agonists stimulation in the absence and presence of Ro320432 are shown in Figures 6.1B and 6.2B, respectively. These data suggest that PKC is only partly involved in the stimulant action of histamine and phenylephrine in the guinea pig ureter smooth muscle.

A



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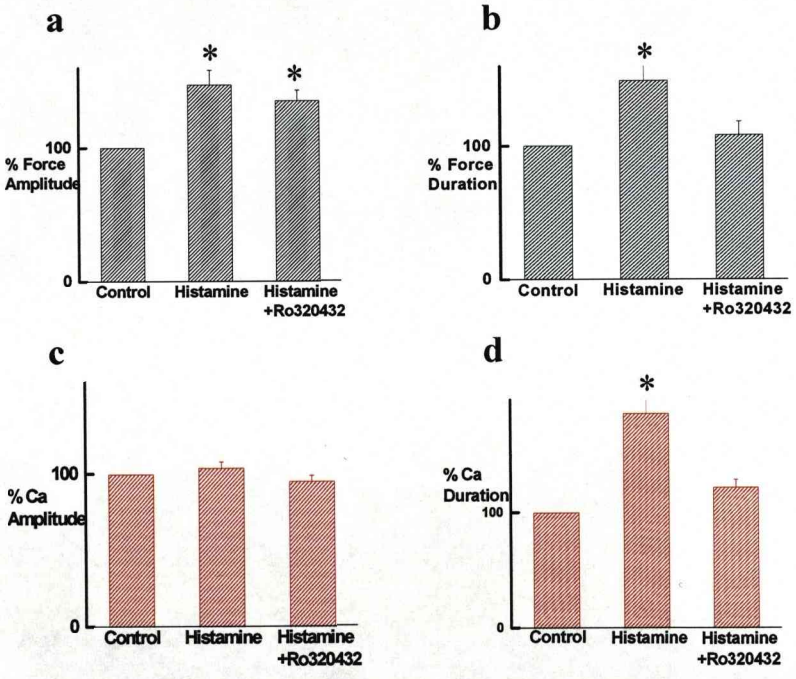
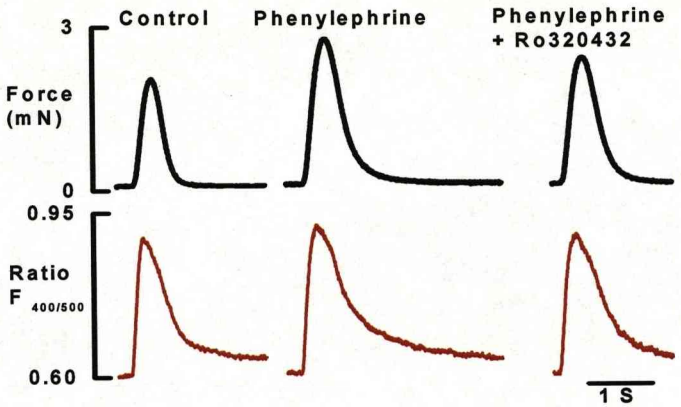


Figurev6.1 A-Individual traces of Ca^{2+} transients and force induced by histamine and histamine in the presence of Ro320432. B-Mean values of amplitude (a) and duration (b) of force and amplitude (c) and duration (d) of Ca^{2+} transients induced by histamine and histamine in the presence of Ro320432 and expressed as percentage of control.

A



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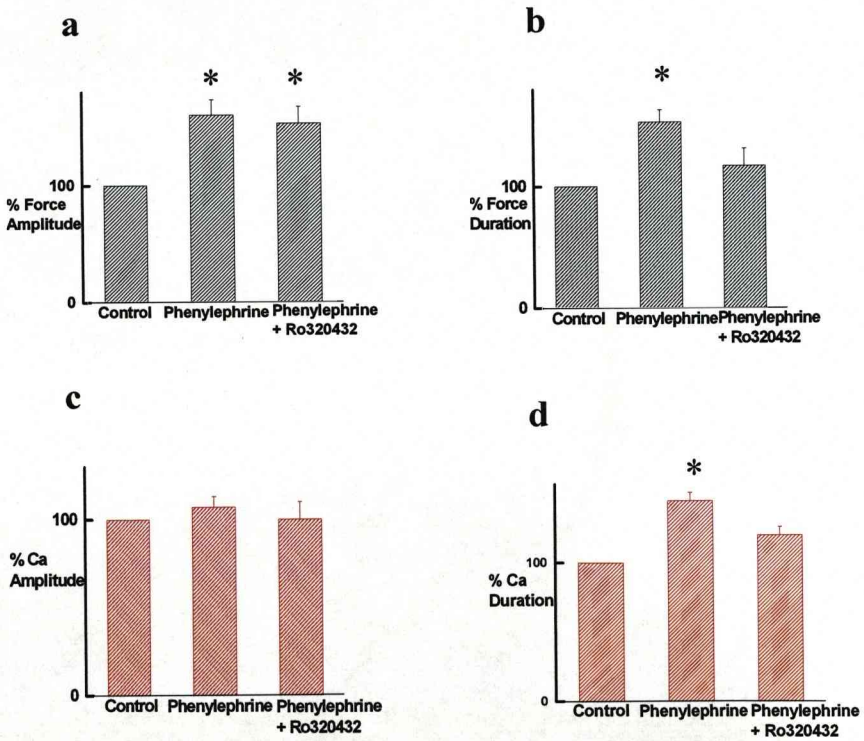


Figure 6.2 A-Individual trace of Ca^{2+} transients and force induced by phenylephrine and phenylephrine in the presence of Ro320432. B-Mean values of amplitude (a) and duration (b) of force and amplitude (c) and duration (d) of Ca^{2+} transients induced by phenylephrine and phenylephrine in the presence of Ro320432 and expressed as percentage of control.

6.4 Discussion

In ureter smooth muscle, the agonists such as histamine and phenylephrine produce stimulant action which is similar to that of PDBu, i.e. all of them produce an increase in duration of the Ca^{2+} transient associated with the plateau component of the action potential (Shuba, 1977(1); Burdyga & Wray, 1999). Thus agonists and PDBu that can prolong the plateau phase will maintain the Ca^{2+} signal at its peak level for longer time which is associated with the increase in the amplitude of force (Shuba, 1977(1); Burdyga & Wray, 1999). In this study, agonists histamine and phenylephrine increased force and the duration of the $[Ca^{2+}]_i$ signal measured at 50% of the peak Ca^{2+} transient which is due to the prolongation of the action potential. This is consistent with the findings of Shuba who showed that histamine has the stimulant effect on action potential and phasic contraction by using double sucrose-gap method (Shuba, 1977(1)). These stimulant effects of histamine and phenylephrine on Ca^{2+} and force in guinea pig ureter are similar to those of PDBu. In vascular smooth muscle, it was shown that application of either agonists such as histamine or PDBu could cause PKC translocation from cytosol to the plasma membrane. Therefore, it is important to know whether PKC plays an important role in the agonists induced modulation of contraction in the guinea pig ureter smooth muscle.

In smooth muscle, agonists can activate specific receptors on cell membrane. Histamine can bind to $H_{1\text{-}4}$-histamine receptors depend on which receptors are

expressed in the tissues while phenylephrine binds to α_1-adrenergic receptor therefore leading to a series of intracellular signal cascades in the smooth muscle cells. Many smooth muscle agonists such as angiotensin II, histamine and α-adrenergic receptor agonists act through receptors coupled to the following mechanism (Hoiting *et al.*, 1996; Challiss *et al.*, 1992). When the agonists activate its receptor on the cell membrane, PLC is activated and leading to hydrolysis of PIP2 to formation of two intracellular messengers, IP_3 and DAG. IP_3 binds to its receptors on SR to trigger the Ca^{2+} release from SR in smooth muscle. In guinea pig ureter smooth muscle, IP_3 receptor is not found on SR but only RyR is expressed (Burdyga & Wray, 1999). IP_3 activity can be excluded in this tissue. Activation of agonists could increase the DAG production and directly activate PKC activity causing PKC translocation from cytosol to the plasma membrane (Langlands *et al.*, 1992; Boterman *et al.*, 2005; Jin *et al.*, 2008). In this study, PKC inhibitor Ro320432 could partly inhibit the stimulant action of both histamine and phenylephrine on Ca^{2+} and force which suggests that PKC is partly involved in the agonist dependent control of contraction in guinea pig ureter smooth muscle. The agonist-induced augmentation of contraction also includes other kinases or proteins such as Rho kinase as found in many other types of smooth muscle, e.g., gallbladder (Seyhan *et al.*, 2005), vascular smooth muscle (Eto *et al.*, 2001; Ratz *et al.*, 2004) and myometrial smooth muscle (Kupittayanant *et al.*, 2001). Activation of smooth muscle contraction by agonists such as phenylephrine was found to translocate RhoA from cytosol to membrane (Gong *et al.*, 1997; Fujihara *et al.*, 1997). PKC is playing a very different role in the agonists-induced contraction in smooth

muscle. Activation of PKC is required for agonists-induced contraction in mouse aorta but not in corpus cavernosal smooth muscle (Jin *et al.*, 2008). PKC is involved in the α_1-adrenergic receptor meadiated contraction in rat tail artery (Sato *et al.*, 2001). In guinea pig lung parenchymal strips, PKC participated in the H_1-receptor mediated contraction via regulation of Ca^{2+} infulx (Leurs *et al.*, 1989). It may be due to that in addition to PKC effect on the MLCP directly, it may have effect on other ion channels or mechanisms.

In conclusion, by using the specific PKC inhibitor Ro320432, we found direct evidence for the partial involvement of PKC in the agonists-induced augmentation of guinea pig ureter smooth muscle contraction by H-histamine receptor and α_1-adrenergic receptor agonists.

Chapter 7

Evidence that PKC is involved in Ca^{2+} sensitization in rat ureter smooth muscle

Chapter 7

Evidence that PKC is involved in Ca^{2+} sensitization in rat ureter smooth muscle

7.1 Introduction

Smooth muscle contraction is controlled by changes in intracellular $[Ca^{2+}]_i$. The contraction can be enhanced by increasing the sensitivity of contractile apparatus to $[Ca^{2+}]_i$ (Kamishima *et al.*, 1992).

The increased cytosolic free Ca^{2+} binds to calmodulin and activates the MLC_{20} phsophorylation which results in smooth muscle contraction (Driska *et al.*, 1981). High K^+ stimulation is a relatively "simple' mechanism of activation of smooth muscle via activation of voltage-dependent Ca^{2+} channels which leads to an increase in cytosolic free Ca^{2+} (Bolton *et al.*, 1979; Ratz *et al.*, 2004). Therefore, it has been used as an experimental model to study the effect of non-receptor activation of smooth muscle contraction.

Muscarinic receptor agonist carbachol could also produce smooth muscle contraction in Ca^{2+}-dependent and Ca^{2+}-independent way (Kamishima *et al.*, 1992). It has been shown that carbachol could cause Ca^{2+} release from SR and contraction in rat ureter smooth muscle by activation of IICR mechanism (Burdyga *et al.*, 1998). In guinea pig ileum, either PDBu or carbachol could cause PKC translocation from the cytoplasm in smooth muscle cells and lead to smooth muscle contraction (Poole *et al.*, 2007). In rat bronchial smooth muscle, carbachol could depolarize cell membrane and activate voltage dependent Ca^{2+} channels (Kamishima *et*

al., 1992). On the other hand, carbachol can induce the contraction by altering the $[Ca^{2+}]_i$ sensitivity which is regulated primarily at the level of MLC_{20} phosphorylation by modulation of MLCP activity (Kitazawa *et al.*, 1991; Kubota *et al.*, 1992). Carbachol binding to G-protein coupled receptors activates PLC which leads to the breakdown of PIP2 leading to production of IP_3 and DAG which activates PKC. PKC could phosphorylate CPI-17 which inhibits MLCP. Therefore PKC can increase MLC_{20} phosphorylation in a Ca^{2+}-independent way in agonist induced contraction (Li *et al.*, 1998; Hartshorne *et al.*, 1998; Eto *et al.*, 2004). KCl does not cause phosphorylation of CPI-17 in the tonic femoral artery or phasic vas deferens in the presence of α-adrenergic receptor blockade (Kitazawa *et al.*, 2000, 2003). Rho A/ROCK was reported to contribute to modulation of MLCP and Ca^{2+} sensitization mechanism in smooth muscle including rat ureter (Shabir *et al.*, 2004; Rats *et al.*, 2004). Rho kinase can also modulate CPI-17 activity (Somlyo AP *et al.*, 2003; Ito *et al.*, 2004).

In Chapter 3, we have described that in rat ureter, PKC is involved in modulation of phasic contraction in a Ca^{2+}-independent manner. To further study this effect we have used several experimental models. First, we have studied the effects of PKC activator PDBu on force-Ca^{2+} relationship in rat ureter smooth muscle stimulated by high K^+ depolarization. In these experiments the effects of PDBu on the amplitude of tonic contraction and its relaxation rate induced by Ca^{2+} free solution have been examined. Secondly, we have used PKC inhibitor Ro320432 to assess possible contribution of PKC to Ca^{2+} sensitization induced by carbachol. In this experiment, the effects of carbachol in Ca^{2+} free solution on Ca^{2+}, MLC_{20} phosphorylation and force in the absence and presence of PKC inhibitor have been investigated.

7.2 Materials and Methods

Simultaneous measurement of calcium and tension

The simultaneous measurement of force and intracellular calcium were described in Chapter 2.

Krebs solution was prepared as described in Chapter 2. The tissue was continuously perfused with Krebs. High K^+ solution was applied for 40 seconds. Carbachol was applied in Ca^{2+}-free solution for 30 seconds.

Measurement of myosin light chain phosphorylation

The measurement of myosin phosphorylation was carried out after separation of the nonphosphorylated and phosphorylated forms of myosin light chain (MLC_{20}) using urea/glycerol-polyacrylamide gel electrophoresis and quantified as detailed in Chapter 2.

Chemicals

All chemicals were purchased from Sigma (Dorset, UK), unless otherwise stated. PDBu was purchased from Calbiochem.

The stock solutions were prepared as following method: PDBu was dissolved in DMSO at a concentration of 1mM. Ro320432 was dissolved in DMSO at a concentration of 5mM.

Carbachol was made up in a stock solution of concentration 10mM. The stock solutions were diluted to the desired concentrations with Krebs solution before starting the experiment.

Statistics

Data was analyzed with t-test; differences between means were assumed to be significant at $P<0.05$. One-Way ANOVA test was applied to test the significant difference ($P<0.05$) between different groups. All values represent mean ± s.e.m; *n* is the number of samples, each one from a different animal.

7.3 Results

7.3.1 The effect of PDBu and Ro320432 on force and Ca^{2+} evoked by EFS and high K^+ in rat ureter smooth muscle

In this study, two experimental models have been used to study the effects of PKC activator PDBu and inhibitor Ro320432 on force and Ca^{2+} in rat ureter-- phasic contraction evoked by EFS (described in chapter 3) and tonic contraction evoked by high K^+ depolarization.

Figure 7.1 (detailed in chapter 3) shows in rat ureter, an increase in the amplitude of phasic contraction produced by PDBu was not associated with changes of the parameters of the Ca^{2+} transients. Relaxation phase of the phasic contraction was slowed down by PDBu with no change in the kinetics of the relaxation decay of the Ca^{2+} transient.

High K^+ solution was applied quickly for 40 seconds. The KCl-induced contraction consisting of initial phasic contraction followed by a sustained tonic component was produced. When tonic contraction reached steady state, the tissue was placed in Ca^{2+}-free solution to induce relaxation in the absence and presence of PDBu.

From Figure 7.2, it can be clearly seen that the tonic component of high K^+ induced contraction was enhanced by PDBu and this stimulant effect can be abolished by Ro320432. The amplitude of tonic contractions in the presence of PDBu was 1.2 ± 0.05 times compared to control (n=9) ($P<0.05$) and Ro320432 brought it back to the control level. The activation of tonic force seen in the presence of PDBu was not associated with an increase in the sustained component of calcium transients (Figure 7.2 A and B).

The effect of PKC on relaxation phase of KCl contraction has been studied. Figure 7.3A shows the superimposed traces of normalized force and Ca^{2+} induced by high K^+ in rat ureter in the absence and presence of 0.1μM PDBu. Figure 7.3 B shows superimposed traces of relaxation phase of both force and Ca^{2+} transient induced by Ca^{2+} free solution which shows that in rat ureter relaxation of force was slowed by PDBu compared to control with no change in the kinetics of the relaxation of the Ca^{2+} transient (Figure 7.3 B and C).

Half-time of relaxation of force in control conditions was 16.3 ± 2.3 s (n=10) and in the presence of PDBu was 23 ± 2 s (n=10) ($P<0.05$). The data obtained strongly suggest involvement of PKC in Ca^{2+} sensitization in rat ureter smooth muscle as an increase in the amplitude of tonic contraction produced by PDBu was Ca^{2+} independent. In addition, marked decrease in the relaxation phase of the tonic contraction induced by Ca^{2+}-free solution seen in the presence of PDBu with no change in the kinetics of the relaxation of Ca^{2+} transient also suggest Ca^{2+} independent effect of PKC on rat ureter contractility.

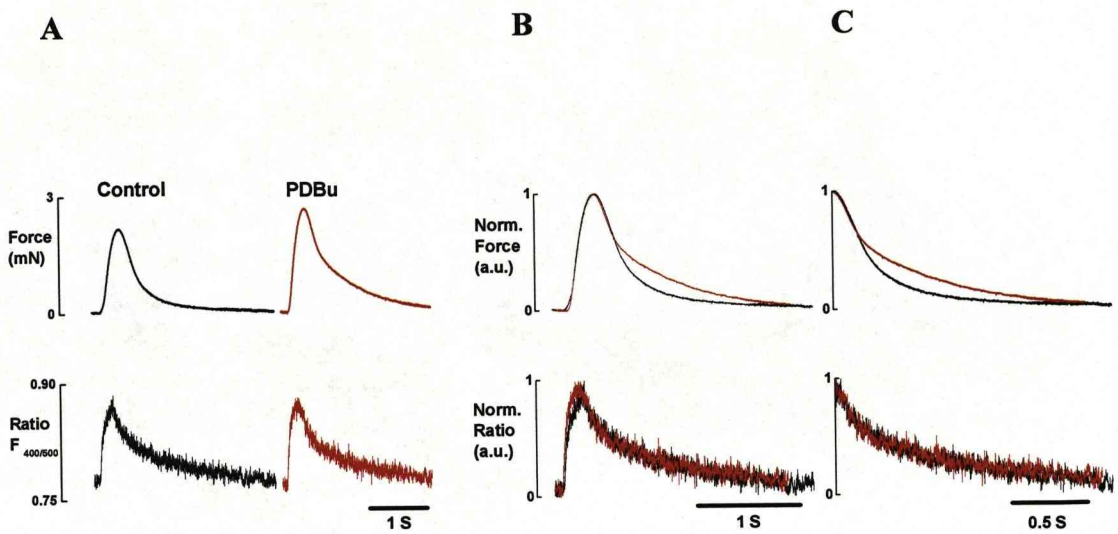
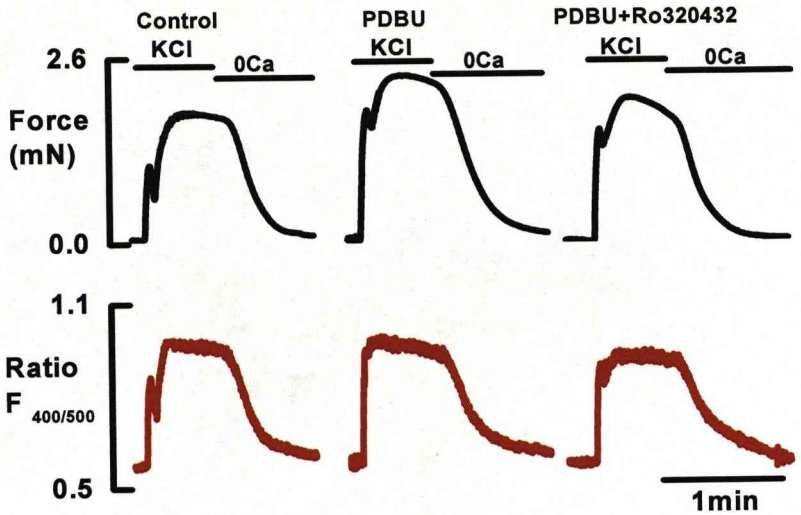


Figure 7.1 The effect of PKC activator PDBu on calcium and phasic contractions evoked by EFS in rat ureter smooth muscle. A- Individual Ca^{2+} transients and phasic contractions of rat ureter smooth muscle recorded in the presence and absence of PDBu; B- Superimposed records from A; C- Superimposed relaxation phase of force and Ca^{2+} transient from B. In this figure, records in red are obtained in the presence of PDBu; Black are controls. Note the relaxation phase of rat ureter phasic contraction is slowed down by PDBu with no change of Ca^{2+}.

A



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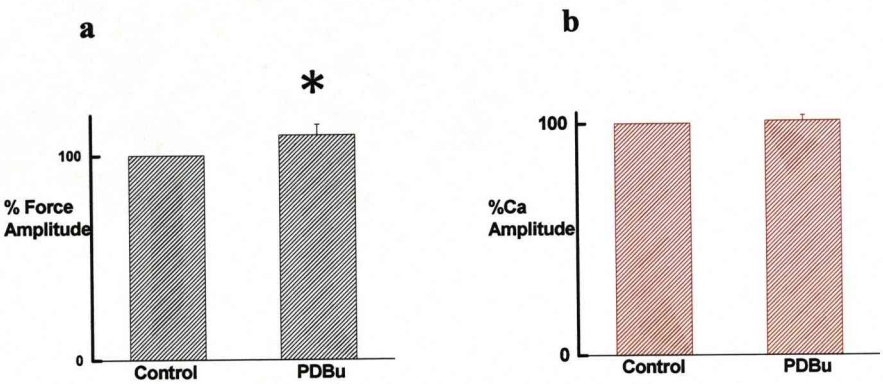


Figure 7.2 The effect of PKC activator PDBu and PKC inhibitor Ro320432 on force and calcium evoked by high K^+ depolarization in rat ureter. A- Force and Ca^{2+} evoked by high K^+ depolarization in control, in the presence of PDBu and PDBu with Ro320432; B- Mean values of amplitude of tonic component (a) and Ca^{2+} transients (b) induced by high K^+ depolarization in the absence and presence of PDBu expressed as percentage of control.

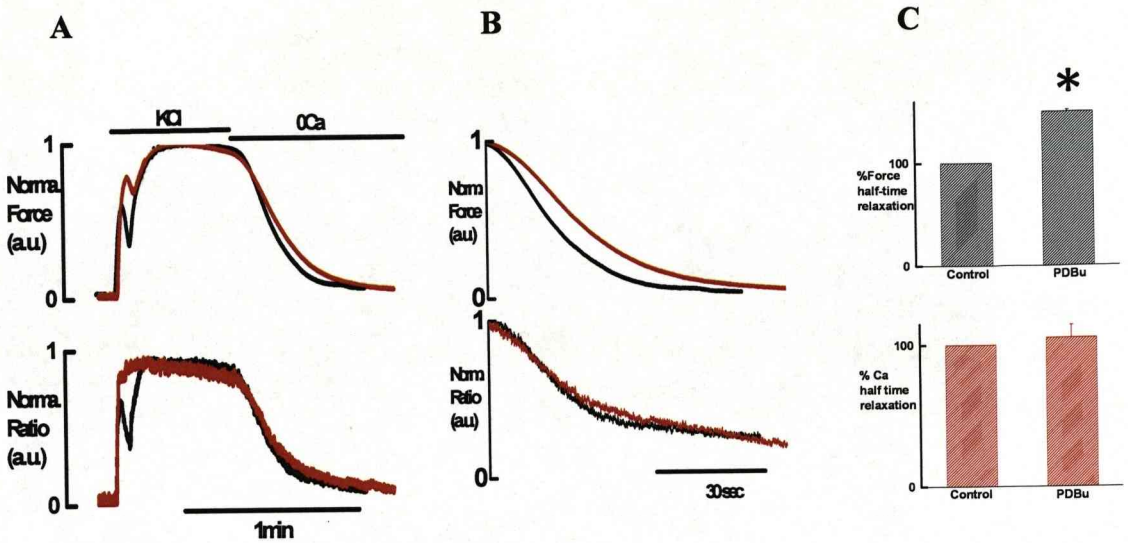


Figure 7.3 The effect of PDBu on the kinetics of force and calcium evoked by high K^+ depolarization in the rat ureter. A- Superimposed records of force and Ca^{2+} transient induced by high K^+ depolarization in rat ureter smooth muscle in the absence and presence of PDBu. B- Superimposed relaxation phase of force and Ca^{2+} transient from A. In Figure A and B, records in red are obtained in the presence of PDBu; Black are controls. C-Mean values of half-time relaxation of force (a) and Ca^{2+} transients (b) induced by high K^+ depolarization in the absence and presence of PDBu and expressed as percentage of control.

7.3.2 The effect of PKC inhibitor Ro320432 on the force, Ca^{2+} transients and myosin light chain phosphorylation evoked by carbachol in Ca^{2+}-free solution

In this study, the role of PKC in agonist induced contraction in rat ureter has been investigated.

The preparation was first stimulated by high K^+ solution for 40 seconds to maximally load the SR with Ca^{2+}. Then, it was placed in calcium free solution for 80 seconds, and stimulated with 100μM carbachol for 30 seconds to induce Ca^{2+} release from SR. The experiments were performed in control condition and in the presence of Ro320432 (10 minutes treatment). Figure 7.4A shows that carbachol could induce a transient Ca^{2+} release from the SR leading to a transient contraction. When PKC was inhibited by Ro320432 the amplitude of force induced by carbachol was decreased to 80 ± 3% of control which was not associated with any change in Ca^{2+} transients (Figure 7.4 B) ($P<0.05$).

The relaxation rate of force induced by carbachol in the presence of Ro320432 was significantly increased while that of the Ca^{2+} transient remained virtually unchanged (Figure 7.5). The $t_{1/2}$ for relaxation of force in control conditions was 13.6 ± 1 s (n=10) and in the presence of Ro320432 was 7 ± 0.5 s (n=10) (Figure 7.5 C) ($P<0.05$). From the phase-plane plots shown in Figure 7.5 B, it can be seen that Ro320432 did not produce any significant effect on force-Ca^{2+} relationship for the rising phase of contraction compared to control. In contrast, the force-Ca^{2+} relationship for the relaxation phase in the presence of Ro320432 significantly shifted to the right which indicated that PKC inhibition produced a Ca^{2+}-independent acceleration of relaxation of force induced by carbachol in rat ureter. Figure 7.5 B shows that the amplitude of force at any given $[Ca^{2+}]_i$ in the presence of Ro320432 was

less compared to control condition. In addition, it can be seen that there is a delay between the rise in $[Ca^{2+}]_i$ and the generation of force, i.e. Ca^{2+} transient peaked before force reached its peak value. Again, in the presence of Ro320432 force peaked earlier and began to prematurely relax despite Ca^{2+} transient remained the same as under control conditions (Figure 7.5 A and B).

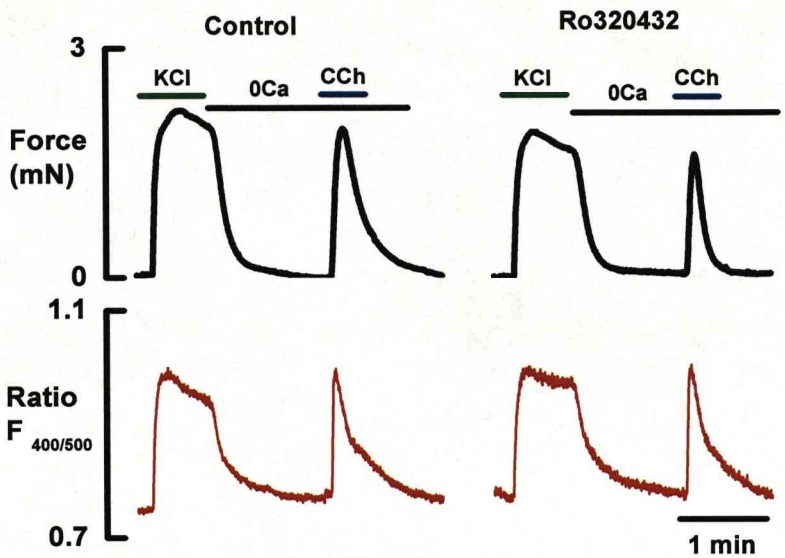
The enhanced relaxation of the carbachol induced contraction with no change in the kinetics of the Ca^{2+} transient suggests that in the presence of PKC inhibition a calcium-desensitization of the contractile machinery to Ca^{2+} is taking place via activation of MCLP leading to a faster dephosphorylation of MLC_{20} in a Ca^{2+}-independent way.

Therefore, in the next set of experiment, the effect of Ro320432 on the myosin light chain phosphorylation levels at different time of development of carbachol induced contraction in the absence and presence of Ro320432 was studied.

The ureteric strips were fast frozen at different times during the rising and relaxation phase of the force (Figure 7.6 B). The myosin light chain phosphorylation level at each point has been measured. Figure 7.6 A shows the representative chemilumigrams of western blots measured during the rising and relaxation phase of carbachol induced contracture (shown by arrows in Figure 7.6 B) in the absence and presence of Ro320432. Figure 7.6 B shows the superimposed scatter plots of force (top panel) and myosin light chain phosphorylation (bottom panel) under control condition and in the presence of 5μM Ro320432 measured at different points during the development of carbachol induced contraction in rat ureter. In the absence of carbachol in Ca^{2+}-free solution there is no detectable level of myosin light chain

phosphorylation. Figure 7.6 B shows that there is no difference in myosin light chain phosphorylation level during the rising phase of the force between control and Ro320432 treated preparation. During carbachol stimulation, myosin light chain phosphorylation quickly reached the maximum level and started to decline to the baseline level. Force was lagging behind MLC_{20} phosphorylation and reached its peak at a time when myosin light chain phosphorylation already declined to about 60-70% of its maximal level. Figure 7.6B shows that force-MLC_{20} phosphorylation relationship during the rising phase of carbachol induced contracture obtained in the presence of Ro320432 was virtually the same as that seen under control conditions. In marked contrast, force-MLC_{20} phosphorylation relationship during the relaxation phase was shifted to the left. Since there was no change in the kinetics of the Ca^{2+} transients induced by carbachol in the absence and presence of PKC inhibition (Figure 7.5 A, bottom trace). These data suggest that force produced by carbachol stimulation in Ca^{2+} free solution is generated in Ca^{2+}-dependent way via activation of Ca^{2+}/Calmodulin dependent MLCK and Ca^{2+} independent way, i.e. Ca^{2+} sensitization via decreasing the activity of MLCP responsible for dephosphorylation and relaxation of the force.

A



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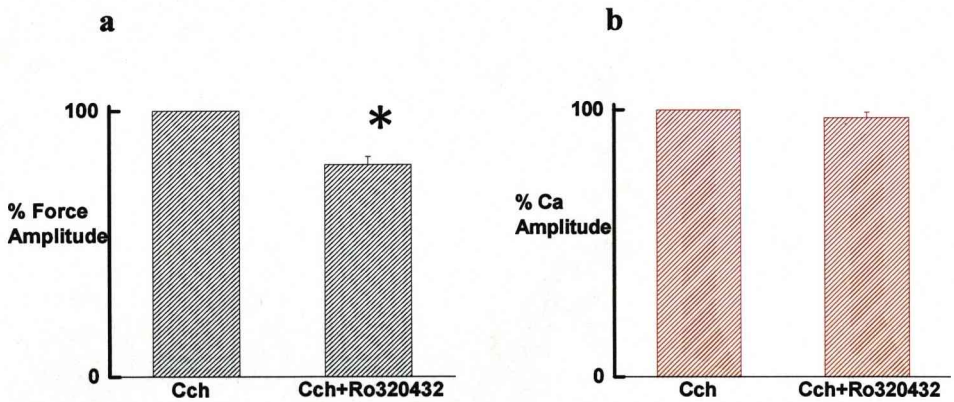

Figure 7.4 The effect of Ro320432 on the force and calcium evoked by carbachol in the rat ureter. A- Force and Ca^{2+} transient evoked by carbachol in the absence and presence of Ro320432 in the rat ureter. B- Mean values of amplitude of force (a) and Ca^{2+} transients (b) induced by carbachol in the absence and presence of Ro320432 expressed as percentage of control.

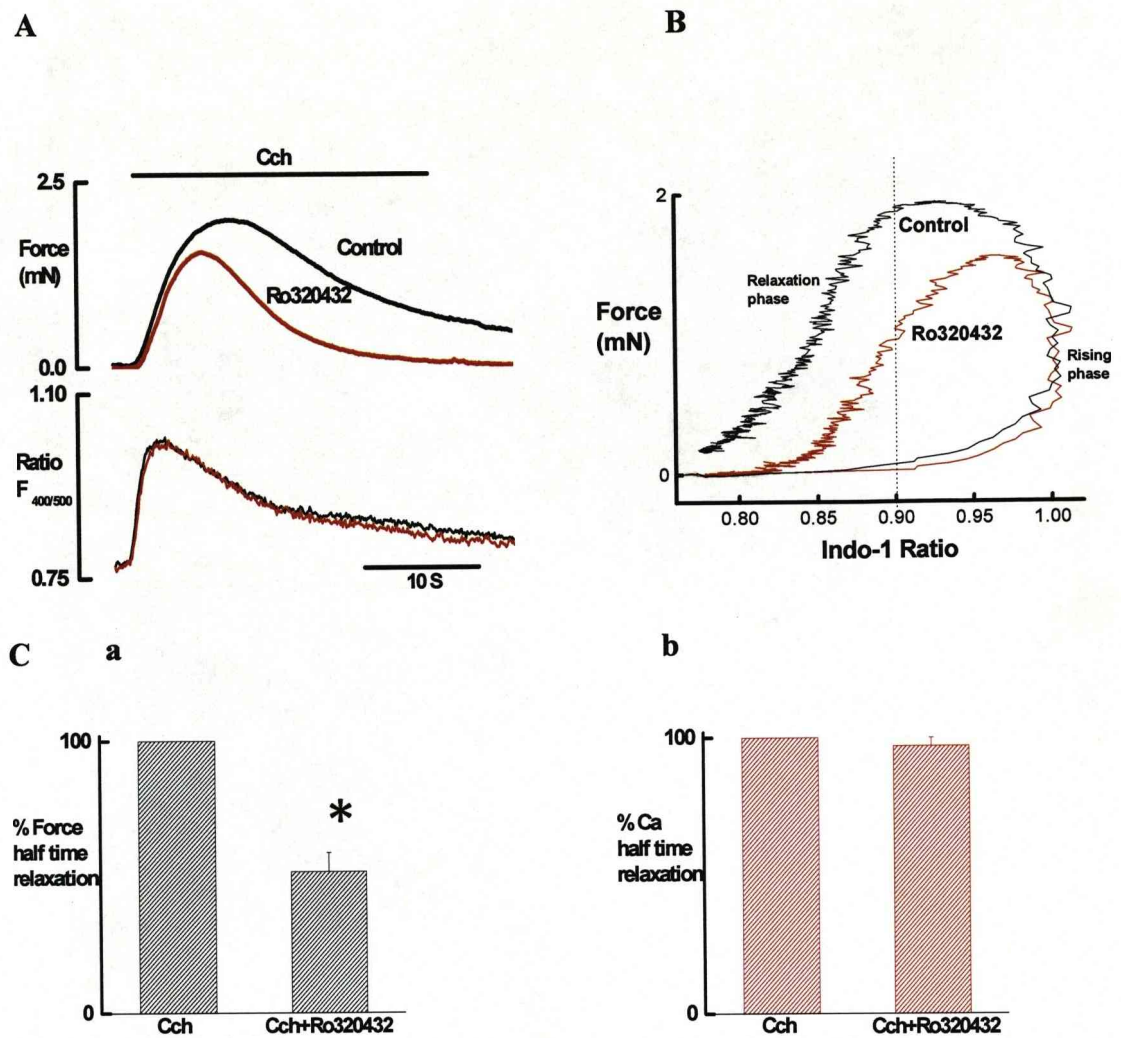

Figure 7.5 The effect of Ro320432 on force and calcium evoked by carbachol in the rat ureter. A- Superimposed records of force and Ca^{2+} transient evoked by carbachol in the absence (black) and presence (red) of Ro320432 in the rat ureter. B- Phase-plane plot showing force-Ca^{2+} relationship during the development of carbachol induced contraction. C- Mean values of half-time relaxation of force (a) and Ca^{2+} transients (b) induced by carbachol in the absence and presence of Ro320432 and expressed as percentage of control.

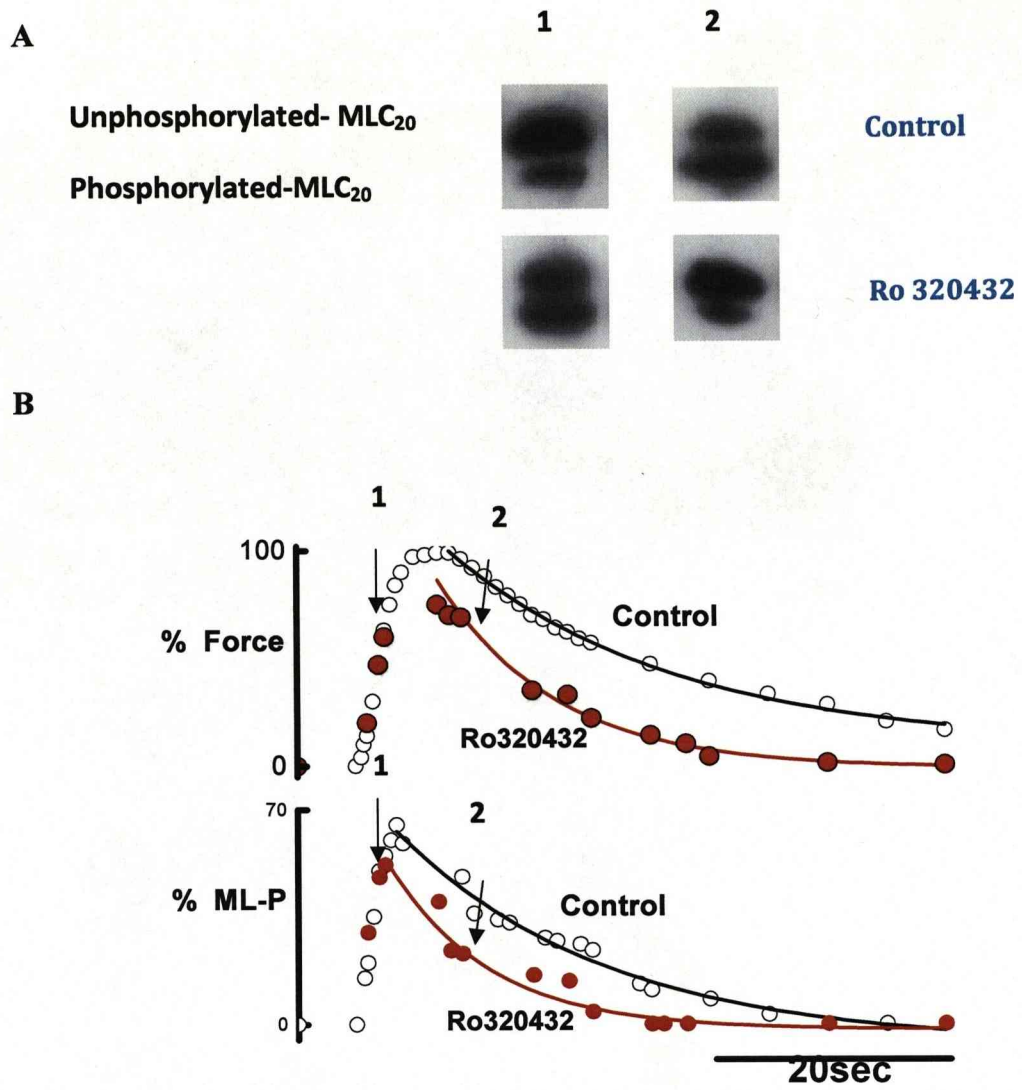

Figure 7.6 Effect of Ro320432 on force and myosin light chain phosphorylation evoked by carbachol stimulation in rat ureter. A-Representative western blots showing phosphorylated and non-phosphorylated bands of MLC_{20} for the relevant points 1 and 2 shown by arrows in B. B- Superimposed scatter plots of force (top trace) and MLC_{20} phosphorylation (bottom trace) of control (black circles), and in the presence of Ro320432 (red circles) induced by carbachol (The data from Figure B were obtained with help of Dr L. Borisova and Dr T. Burdyga).

7.4 Discussion

In smooth muscle, the increased intracellular Ca^{2+} binds to calmodulin and this complex activates the myosin light chain kinase which phosphorylates the myosin light chain at Ser19 (Hartshorne *et al.*, 1987). It is required for activation of the actin-activated myosin ATPase of smooth muscle contraction (Hartshorne *et al.*, 1981). The level of MLC_{20} phosphorylation can also be modulated in Ca^{2+} independent way via modulation of myosin light chain phosphatase activity. Several kinases have been shown to contribute to modulation of the activity of MLCP in smooth muscle. PKC has been suggested to play an important role in modulating the activity of MLCP (Somlyo & Somlyo, 1994). Activation of PKC can inhibit MLCP and therefore leads to an increase in smooth muscle contraction.

To examine possible role of PKC in control of Ca^{2+} sensitivity in rat ureter smooth muscle contraction, we have investigated the effects of PKC activation and inhibition on force-Ca^{2+} relationship using different modes of stimulation such as EFS, high K^+ depolarization and carbachol.

Our data suggest that in rat ureter smooth muscle, the effect of PKC activation appears to affect force by sensitizing contractile machinery to Ca^{2+} irrespective of the modes of stimulation. When ureter smooth muscle was treated with 0.1μM PDBu, the amplitude of the phasic contraction induced by EFS and amplitude of sustained component of high K^+ induced contractions were increased with no change in $[Ca^{2+}]_i$. Measurement of half-time of relaxation phase of phasic contraction and tonic contraction both shows that PDBu could slow down the relaxation without change of intracellular Ca^{2+} level. These findings are similar to those obtained in other types of smooth muscle in which activation of PKC by

agonists activate force without a corresponding change in intracellular Ca^{2+} (Andrea *et al.*, 1992). Therefore, we could conclude that PKC regulates rat ureter smooth muscle contraction in Ca^{2+}-independent way and is likely to be involved in Ca^{2+}-sensitization mechanism by affecting the activity of MLCP.

PKC has been shown to play an important role in control of contraction by affecting MLCP - the process called Ca^{2+} sensitization in several types of smooth muscle (Kitazawa *et al.*, 1999; Takuwa, 1996; Shirao *et al.*, 2002). It was shown that activation of PKC phosphorylated CPI-17 at Thr38 thus increasing its inhibitory potency of MLCP over 1000 fold (Eto *et al.*, 1997; Hayashi *et al.*, 2001). Therefore activation of PKC could lead to an increase in force generated in smooth muscle without change of intracellular Ca^{2+}.

In tonic smooth muscles, agonists binding to G-protein coupled receptor leads to hydrolysis of PIP2 which then leads to formation of IP_3 and DAG. IP_3 binds to IP_3 receptor causing Ca^{2+} release from SR and thereby producing an initial intracellular calcium transient accompanied by force generation (Merkel *et al.*, 1991; Somlyo & Somlyo *et al.*, 1994).

In rat ureter, PKC is involved in agonist induced modulation of contraction in Ca^{2+}-independent way. Application of PKC inhibitor Ro320432 could inhibit the cabachol induced contraction while the level of Ca^{2+} remained unaltered. Furthermore, Ro320432 increased the rate of relaxation of carbachol induced contraction with no change in the kinetics of Ca^{2+} transients which suggests that PKC is involved in the agonist induced contraction in Ca^{2+}-independent way.

Many studies performed on tonic smooth muscles suggested that activation of PKC could inhibit the MLCP activity thereby increasing the phosphorylation level of MLC_{20} (Budzyn *et al.*, 2006). In case of carbachol stimulation in rat ureter, myosin light chain phosphorylation level can be directly correlated with force because Ca^{2+} signal is not altered between control and Ro320432 treated preparation. From the phase-plane plot, it can be clearly seen that force-Ca^{2+} relationship for the preparation treated with Ro320432 during relaxation phase is shifted to the higher level of $[Ca^{2+}]_i$ suggesting desensitization of the contractile activity to Ca^{2+}. These data are in good agreement with a left shift of force-MLC_{20} phosphorylation relationship seen in the presence of Ro320432 during carbachol stimulation. The decreased amount of force generated in the presence of Ro320432 is due to the decreased myosin phosphorylation level. The acceleration of relaxation of force produced by Ro320432 is accompanied by the acceleration of dephosphorylation of the myosin light chain. This suggests that inhibition of force is due to an increased activity of MLCP. Inhibition of PKC increases MLCP activity leading to acceleration of dephosphorylation of myosin light chain during the relaxation phase of agonist-induced contraction. Collectively, the data obtained strongly suggest that the main mechanism underlying direct stimulant action of PKC by PDBu involves Ca^{2+}-independent sensitization of contractile machinery and that this mechanism is partly involved in control of the stimulant action of agonists in rat ureter smooth muscle.

Chapter 8
Summary

Chapter 8

Summary

The main aim of the present work was to investigate role of PKC in control of excitation-contraction coupling in the guinea pig and rat ureter smooth muscle. The main finding of this study was that PKC was expressed and functionally important in control of contraction in the ureter of both species. However, expression, distribution and the mechanisms of stimulant action of PKC activation on ureter smooth muscle were species dependent.

PKC activator PDBu and inhibitor Ro320432 were used to investigate the functional role of PKC in control of phasic contraction in the guinea pig and rat ureter smooth muscle. Application of 0.1μM PDBu produced a significant increase in the amplitude and duration of phasic contraction which was accompanied by an increase in the duration of the Ca^{2+} transient in guinea pig ureter while in rat ureter an increase in the amplitude of phasic contraction by PDBu was Ca^{2+} independent. PKC specific inhibitor Ro320432 at concentration of 5μM could fully reverse the stimulant effects of PDBu in both guinea pig and rat ureter smooth muscle. PDBu prolonged the relaxation time of the phasic contraction in rat but not in guinea pig ureter and it was not associated with any change in the kinetics of the Ca^{2+} transient. Overall, we found that PKC was involved in regulation of phasic contraction in a Ca^{2+}-dependent way in the guinea pig and in a Ca^{2+}-independent way in the rat ureter smooth muscle.

The different role of PKC involved in modulation of intracellular Ca^{2+} in two species might be due to the different expression and distribution patterns of various PKC isoforms. In the guinea pig ureter smooth muscle PKC α, β and δ are expressed. In contrast, PKC α is present in rat ureter smooth muscle.

8.1 Mechanism of the stimulant action of PKC activation in the guinea pig ureter

Early electrophysiological studies demonstrated that action potential in the guinea pig ureter smooth muscle consists of the spike and plateau component (Branding *et al.*, 1983; Burdyga & Wray, 1999). Patch clamp studies revealed that there are two major ionic currents responsible for generating the complex action potential in the guinea pig ureter smooth muscle: inward Ca^{2+} current and outward K^+ current. Ca^{2+} current is produced by activation of L-type voltage gated Ca^{2+} channels and outward K^+ current by large conductance K^+ channels (BK_{Ca}) (Lang, 1989; Imaizumi *et al.*, 1989). Recently it was shown that BK_{Ca} channels in the guinea pig ureter were activated by Ca^{2+} sparks (Burdyga & Wray, 2005).

8.2 Functional role of PKC in guinea pig ureter smooth muscle contraction

We have found that in guinea pig ureter smooth muscle activation of PKC by PDBu prolonged the duration of the plateau component of the action potential. This can be achieved by either activation of L-type Ca^{2+} channels or direct or indirect (via inhibition of Ca^{2+} sparks) inhibition of BK_{Ca} channels.

8.2.1 Evidence that Ca^{2+} sparks/STOCs coupling mechanism is not involved in the stimulant action of PKC

Ca^{2+} sparks/STOCs coupling mechanism plays a very important role in setting the refractory period and control of the duration of the action potential in guinea pig ureter smooth muscle (Burdyge & Wray, 2005; Borisova *et al.*, 2007). Inhibition of Ca^{2+} sparks or their target BK_{Ca} channel by TEA prolonged the duration of the action potential (Burdyga *et al.*, 1995; Borisova *et al.*, 2007) which is associated with an increase in the duration of the Ca^{2+} transient leading to an increase in the amplitude and duration of force (Burdyga & Wray, 1999). In the present study, we have found that in guinea pig ureter smooth muscle PDBu produced marked increase in the amplitude and duration of force accompanied by an increase in the duration of the Ca^{2+} transient under experimental conditions when the SR function was fully blocked by CPA leading to inhibition of Ca^{2+} sparks or when BK_{Ca} channels were blocked by TEA. In fact, the stimulant action of PDBu under these experimental conditions was increased. Therefore, the stimulant action of activation of PKC on action potential is not due to inhibition of Ca^{2+} sparks/STOCs coupling mechanism in the guinea pig ureter smooth muscle.

In agreement with these data we have also found that PKC activator PDBu actually increased the frequency of spontaneous Ca^{2+} sparks therefore could be expected to activate the Ca^{2+} sparks/STOCs coupling mechanism in the guinea pig ureter smooth muscle. If the effect of PKC on Ca^{2+} sparks/STOCs coupling mechanism was the only

mechanism involved in regulation of guinea pig ureter smooth muscle contraction, the duration of the plateau component of the action potential could be decreased by PKC activation. However, our data clearly show that activation of PKC increased the duration of action potential and that it is not involved in inhibition of Ca^{2+} sparks/STOCs coupling mechanism.

8.2.2 Is Na^{+}-Ca^{2+} exchanger involved?

It was shown that both extracellular Na^{+} and Ca^{2+} contributed to the generation of the plateau component of the action potential in the guinea pig ureter smooth muscle (Shuba, 1977(2)). Removal of either extracellular Na^{+} or Ca^{2+} abolished plateau component of the action potential. There is no voltage-dependent Na^{+} channel in ureter smooth muscle and it was suggested that Na^{+}-Ca^{2+} exchanger might contribute to this effect (Kuriyama *et al.*, 1967; Shuba, 1977(2); Aaronson & Benham, 1989; Sui *et al.*, 1997). Removal of extracellular Na^{+} caused significant decrease in the amplitude and duration of force associated with Ca^{2+} transients which is due to the abolishment of the plateau component of action potential as was found earlier (Shuba, 1977(2); Brading *et al.*,1983; Imaizumi *et al.*, 1989; Burdyga *et al.*, 1999). Our data clearly show that in Na^{+}-free solution PDBu still produced strong stimulant effect on phasic contraction and intracellular Ca^{2+}. These data suggest that the stimulant effects of PDBu on Ca^{2+} and force are not associated with the targeting Na^{+}-dependent mechanism.

In agreement with these suggestions we also report lack of any effect of PDBu on Ca^{2+} and force induced by Na^{+}-Ca^{2+} exchanger in Ca^{2+}-entry mode in Na^{+}-loaded ureter using protocols designed by Dr. T. Burdyga and his colleagues (Aickin *et al.*, 1984; Burdyga & Magura, 1987; Lamont *et al.*, 1999). Therefore, the data obtained suggested that the Na^{+}-Ca^{2+} exchanger or any other Na^{+}-dependent mechanisms controlling the duration of the plateau component of the action potential are not involved in the stimulant action of PDBu on the action potential in the guinea pig ureter smooth muscle.

8.2.3 PKC and L-type Ca^{2+} channel

One could suggest that the stimulant action of PKC activation in the guinea pig ureter smooth muscle could involve modulation of voltage gated L-type Ca^{2+} channels. Preliminary experiments performed with help of Dr T. Burdyga using path clamp technique showed that PDBu increased the amplitude of the inward Ca^{2+} current (data not shown). However, more systemic studies were precluded by lack of time.

8.2.4 Is PKC involved in agonist effects?

PKC is involved in the stimulant action of agonists in different types of smooth muscle (Langlands *et al.*, 1992; Collins *et al.*, 1992; Boterman *et al.*, 2005; Jin *et al.*, 2008). In the guinea pig ureter smooth muscle, histamine and phenylephrine have been shown to have stimulant effect on force via prolongation of the plateau component of the action potential (Shuba, 1977(1)). Using PKC inhibitor Ro320432

in concentration which fully reversed stimulant action of PDBu we have found that stimulant action of either histamine or phenylephrine was only partly inhibited by PKC inhibitor. It suggests these agonists in addition to PKC also activate some other mechanisms which contribute to their stimulant action. PKA, Rho-kinase and other likely kinases could also be involved. However, possible role of the other kinases involved in the stimulant action of agonists in the guinea pig ureter smooth muscle was outside scope of this project.

8.3 PKC and rat ureter smooth muscle

In contrast to the guinea pig ureter, in rat ureter smooth muscle, activation of PKC by PDBu produced stimulant effect on force without affecting parameters of the Ca^{2+} transients.

Our data strongly suggest that the main mechanism involved in the stimulant action of PDBu in rat ureter smooth muscle involves Ca^{2+} sensitization of the contractile machinery to Ca^{2+}. Ca^{2+} sensitization was discovered in a number of smooth muscles and both PKC and Rho-kinase were shown to be involved (Somlyo & Somlyo, 1994; Kitazawa *et al.*, 2000; 2003; Sakai *et al.*, 2007; Mizuno *et al.*, 2008). Both kinases were shown to inhibit myosin light chain phosphatase and thus producing additional increase in the level of myosin light chain phosphorylation and force at a constant level of $[Ca^{2+}]_i$ (Kitazawa *et al.*, 2000, 2003; Eto *et al.*, 2004; Shabir *et al.*, 2004; Ratz *et al.*, 2004;Sakai *et al.*, 2007). The fact that relaxation of phasic or tonic contraction

by PDBu with no change in the kinetics of Ca^{2+} transients as well as potentiation of tonic component of high K^+ contracture at the same $[Ca^{2+}]_i$ strongly suggests that PKC also potentiates force in rat ureter smooth muscle via Ca^{2+} sensitization mechanism.

Our data also indicate that PKC is involved in Ca^{2+} independent stimulant action of carbachol. Indeed, inhibition of PKC by Ro320432 markedly decreased the amplitude and accelerated the relaxation of carbachol induced contracture which correlated well with an increase in the rate of myosin light chain dephosphorylation in rat ureter smooth muscle. These changes in force and myosin light chain phosphorylation were observed with no change in the parameters of the Ca^{2+} transient associated with Ca^{2+} release from the SR. Our data suggest that carbachol binding to its receptor results in production of IP_3 which activates Ca^{2+} release from the SR. Ca^{2+} binds to calmodulin and activates Ca^{2+}/calmodulin dependent myosin light chain kinase which phosphorylates myosin light chains triggering cross bridge cycling leading to contraction. DAG activates PKC which inhibits activity of MLCP and this results in further increase in the level of myosin light chain phosphorylation at the same level of $[Ca^{2+}]_i$ leading to additional increase in the contractile response, i.e. Ca^{2+} sensitization pathway. CPI-17 can be phosphorylated by PKC and increase its strong inhibitory effect on MLCP. The inhibitory potency of the phosphorylated form of CPI-17 can be increased more than 1,000 times by PKC activation (Eto *et al.*, 1997; Hayashi *et al.*, 2001).

Our data clearly indicate that guinea pig and rat ureter express different isoforms of PKC and this could be one of the possible reasons of the species dependent effects observed. Guinea pig predominantly expressed PKC β and δ while rat ureter predominantly expressed PKC α. Whether this difference in PKC expression can explain the difference in the stimulant action of PKC activation in ureter smooth muscle requires additional experiments by using other experiment approaches.

8.4 Future work

To investigate the mechanism of the stimulant action of PKC in guinea pig ureter, the effects of PKC activation on the ionic currents in voltage clamped ureteric myocites should be investigated by using the whole cell voltage clamp technique. Specific inhibitors of different PKC isoforms or RNAi could be used to identify functional role of different PKC isoforms in the ureter of both species. Expression and functional role of PKC in mice ureter could be investigated which would allow to use PKC KO mice as experimental models. In rat ureter phosphorylation of MYPT1 by PKC via CPI-17 should be investigated to fully understand the mechanism of Ca^{2+} sensitization.

Conclusions

1. Expression and functional role of PKC in the guinea pig and rat ureter smooth muscle have been studied.

2. In the guinea pig ureter three PKC isoforms α, β and δ have been detected. PKC α was present in the smooth muscle but was more abundant in urothelium; PKC β was present in both smooth muscle and urothelium. PKC δ was a dominant isoform expressed in the guinea pig ureter and was detected only in the smooth muscle.

3. In the rat ureter only two PKC isoforms α and β have been detected. PKC α was present in both smooth muscle and urothelium; PKC β was detected only in urothelium.

4. Direct activation of PKC by PDBu produced stimulant action on ureter smooth muscle of both species. However in the guinea pig ureter it was associated with an increase in the duration of the Ca^{2+} transient which was correlated with an increase in the duration of the plateau component of the action potential. Neither Ca^{2+} sparks/STOCs coupling mechanism nor Na^{+}-Ca^{2+} exchanger was involved.

5. In the rat ureter smooth muscle direct activation of PKC by PDBu produced an increase in the amplitude of phasic contractions with no change in parameters of the Ca^{2+} transient suggesting that Ca^{2+} sensitization mechanism is the main mechanism.

6. PKC inhibitor Ro320432 fully reversed stimulant action of PDBu in the ureter smooth muscle of both species and significantly decreased the stimulant action of agonists.

7. The data obtained suggest that in the guinea pig ureter smooth muscle stimulant action of PKC on the duration of action potential is likely to be caused by stimulation of the inward Ca^{2+} current via L-type Ca^{2+} channels.

8. In the rat ureter Ca^{2+} sensitization associated with inhibition of MLCP is the major mechanism underlying the stimulant action of PKC activation on the contractile activity.

9. In the guinea pig ureter, histamine and phenylephrine produced stimulant action by increasing the duration of the Ca^{2+} transient similar to PDBu. PKC inhibitor Ro320432 partly reduced the stimulant action of these agonists which suggests involvement of PKC.

10. In the rat ureter stimulant action of carbachol involved Ca^{2+} sensitization mechanism via inhibition of MLCP. PKC partly contributd to this stimulant effect.

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